Investigation of Sarcopenia in a Murine Model:

Symptoms of Age-Related Neuromuscular Decline

and

Resistance Training Intervention

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Theodore G. Graber

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Dedication

I dedicate this work to my wife, Kathleen Bowman-Graber, who stood alongside me throughout the difficult years that my returning to school brought to our family, “We did it!”

Abstract

The world population demographic is rapidly aging. With advancing age comes the onset of age-related diseases and syndromes such as sarcopenia, the age-associated loss of muscle mass and strength. Sarcopenia leads to a multitude of adverse outcomes including a reduced quality of life, increased mortality, functional disability, and eventual loss of independence. Currently no cure for sarcopenia exists and its etiology is still largely undefined. Thus, there is a need for animal models for preclinical investigation of novel interventions. The overall purpose was to first investigate, characterize, and describe the neuromuscular healthspan of the C57BL/6 mouse, a common animal model of aging, and then to subsequently create a treatment model for sarcopenia by producing and validating a voluntary resistance training protocol for mice. The first research chapters (2 and 3) investigate and define the neuromuscular healthspan and the age-related decline of contractile parameters in the mouse. Chapter 2 outlines the neuromuscular healthspan scoring system, a composite score consisting of two functional measures combined with *in vitro* maximum isometric force of the extensor digitorum longus (EDL). This composite outcome measurement increased the power to detect change beyond the capacity of the individual component measures alone. Chapter 3 examines unique aspects of contractile velocity and power production in the soleus and EDL, revealing that age has a greater effect on concentric contraction performed at higher percentages of maximum force. Because the only consensus treatment for sarcopenia is resistance exercise, in Chapter 4 a mouse model of voluntary resistance training was designed and validated. The protocol was designed using human principles of weight training and was assessed with a comprehensive battery of outcome measurements. The outcomes were selected to test whether the mice had the same type of adaptations as would be observed in humans undergoing a similar training intervention. In Chapter 5, the resistance training protocol was applied to a cohort of aged mice to test if signs of anabolic resistance would be detected. Overall, the thesis tells the story of age-related neuromuscular dysfunction that can be partially rescued though exercise and creates a novel preclinical animal model of voluntary resistance training.

Table of Contents **Page**

List of Tables (By Chapter[1]) **Page**

Chapter 2

Chapter 3

Chapter 4

Chapter 5

[1] All Tables are renumbered by chapter to preserve the integrity of the manuscript format.

List of Figures (By Chapter[2]) **Page**

Chapter 1

Chapter 2

[2] All Figures are renumbered by chapter to preserve the integrity of the manuscript format.

List of Figures (By Chapter[3]) Continued **Page**

Chapter 3

[3] All Figures are renumbered by chapter to preserve the integrity of the manuscript format.

List of Figures (By Chapter[4]) Continued **Page**

Chapter 4

[4] All Figures are renumbered by chapter to preserve the integrity of the manuscript format.

List of Figures (By Chapter[5]) Continued **Page**

Chapter 5

[5] All Figures are renumbered by chapter to preserve the integrity of the manuscript format.

List of Figures (By Chapter[6]) Continued **Page**

Chapter 5

[6] All Figures are renumbered by chapter to preserve the integrity of the manuscript format.

Introduction

The global population of older adults continues to grow at an exponential rate. In 1950 there were 205 million persons over the age of 60, in 2010 this population was estimated to be almost 810 billion, and by 2050 it is estimated that over 2 billion people will more than 60 years old (UNDESA, 2013). At the same time the birthrate has more or less flat-lined (UNDESA, 2013), thus there will be fewer younger people per older individual than in the past. The United States had 40.3 million people over 65 in 2010, or 13% of the population, which is about 12 times as many at those ages as were alive in 1900, about 4% of the total (US Census Bureau, 2011). By 2050, the US Census Bureau estimates that, at current rates of growth, the over-65 population will stand at 20.9%. About 38% of these older individuals in 2010 presented with at least 1 form of disability (mobility and activities of daily living challenges being the most common). Thus, this new demographic wave of older adults, many of whom will be potentially disabled and/or presenting with numerous comorbidities such as diabetes, heart disease, and cognitive dysfunction (or other age related conditions) will be supported by a proportionately sinking level of younger workers in the tax base. Obviously this will strain the economic capacity, and the will, of our society as we attempt to provide for the needs of this population. Addressing health concerns of the elderly now, before it is too late, with an eye towards designing invention strategies to reduce the debilitating effects of chronic conditions, is an absolute imperative.

With increasing age comes the onset of sarcopenia. Sarcopenia, the age-related loss of muscle mass and strength, is a major health concern that contributes to the onset of frailty (Marzetti, 2006; Sundell, 2011). Frailty is a multifactorial syndrome that has been characterized by low functional reserve, loss of muscle mass and strength, decreased mobility, increased weakness, and low energy expenditure (Fried, 2001). Sarcopenia (and by extension frailty) leads to falls, a deteriorating quality of life, difficulty performing activities of daily living, eventual loss of independence, and is a major predictor of poor prognoses after procedures and increased mortality (Landi, 2012; Rolland, 2008; Breen, 2011; Janssen, 2002). Loss of independence is a huge blow to the quality of life of the individual and a huge cost for society, so to find strategies to extend the independent life of seniors is critical. Combined with the high disability rates and often multiple comorbidities of the elderly, sarcopenia must be seen as major health challenge as the older population continues to grow. Therefore, it is in our best interest as a society to invest resources into investigating intervention strategies to mitigate sarcopenia and, by extension, frailty.

At present there is no cure for either sarcopenia or frailty. Exercise, however, is an accepted intervention that has been demonstrated to improve these conditions. Progressive resistance training with weights is a well-accepted intervention to mediate sarcopenia in humans (Marini, 2008; Fiatarone, 1990;

Pillard, 2011). This form of exercise reduces age-related myofiber apoptosis (Marzetti, 2006), induces gains in strength and muscle mass (Fiatarone, 1990; Latham, 2004), improves functional outcome measurements (Marini, et al. 2008; Capodaglio, 2007; Fiatarone, 1990), and confers positive metabolic changes (Sundell, 2011). While the strategy of resistance training has many benefits, this form of intervention does not totally reverse sarcopenia. In fact, many other interventions show some benefit (e.g., hormonal, nutritional, ergogenic and pharmaceutical supplementation); however, total mitigation of sarcopenia has not been achieved with a single therapy. Most likely multiple cellular mechanisms are responsible for the inability of the individual therapies to be effective (e.g., lowered rates of protein synthesis, blunted response to amino acid feeding, impaired cellular signaling) (Narici, 2010). Anabolic stimuli, such as exercise or nutrient intake, have been shown to have a lessened effect in older individuals, and this has been termed anabolic resistance.

There are multiple physiological levels and signaling pathways that potentially contribute to anabolic resistance (Burd, 2013). It had long been presumed that there was an overall reduced capacity, in response to anabolic stimuli, for protein synthesis in older animals (Fry, 2011, Koopman, 2009). This has been tested by examining nutrient signaling and response to exercise, both of which resulted in reduced adaptations with age (Kumar, 2009; Degens, 2003). Recently, some evidence has emerged that challenged this paradigm. It may be that older

individuals have the same capacity for protein synthesis after feeding (Symons, 2011). In Symons, et al. older and younger adults both increased protein synthesis 108% from baseline after an acute bout of resistance training followed by a high protein meal (340 g of beef). It is also possible that older individuals may require a larger minimum signal to initiate transcription and translation at the same level as younger adults. In one recent example, a study of muscle protein synthesis rates after protein feeding found that more protein was required in the older adults to achieve the same level as the younger subjects (Moore, 2015). In a study of maintaining training adaptations, it was found that younger adults could maintain benefits received from exercise with lower dose of maintenance exercise than the older adults (Bickel, 2011). Acute bouts of resistance training have been shown to result in a reduced level of phosphorylation of key protein mTOR synthesis pathway proteins (p70S6K, for example) in older subjects (Fry, 2011). However, a study combining resistance training and post-exercise protein feeding found that phosphorylated p70S6K (marker of protein translation) was elevated in both an older and younger cohort at the acute level, but was blunted in the older group after a training program (Farnfield, 2012). This is in contrast to previous research showing no added benefit of protein ingestion combined with resistance exercise in the elderly (Verdijk, 2009). Current consensus in the field is that more research is needed to understand potential etiology of the various forms of anabolic resistance and to develop therapies.

The systemic physiological environment may be less conducive towards muscle hypertrophy in the elderly, thus contributing to anabolic resistance. One example is the hormonal changes, as evidenced by the decline in testosterone and growth hormone with age (both potent anabolic agents) (Sipila, 2013; Valenti, 2010; Gray, 1991; Van den Beld, 2000). Meta-analysis of testosterone replacement therapy has demonstrated increasing strength and mass with treatment (Ottenbacher, 2006; Neto, 2015). Much research into hormone replacement therapy has been done and this continues to be controversial with the risk/benefit analysis ongoing and new therapies being tested (Jusuja, 2014; Garnick, 2014). A second example is age-associated global inflammation, which has been linked to reduced capacity to respond to exercise (Merritt, 2013). Many of the repair mechanisms in muscle that would be activated after a vigorous exercise routine rely upon inflammatory signaling to activate response (Evans, 1991). Thus, a high level of background noise (global elevation of inflammatory signaling) may obscure the normal repair signaling response, thus limiting adaptation (Jo, 2012). Disruption and impairment of cellular processes involved in nutrient sensing, signaling cascades for protein translation/synthesis, and satellite cell biology also could be contributors to lower anabolic response with age. Chapter 1 Background Section F. Anabolic Resistance Etiology provides a more in depth discussion of some potential mechanisms of anabolic resistance.

Designing interventions that have the potential to target the age-impaired anabolic processes may synergize with the positive benefits of resistance training. Combining timed nutrient ingestion or other interventions (e.g. ergogenic aids such as creatine monohydrate) with resistance training to overcome anabolic resistance and improve exercise response in older individuals may successfully prevent, attenuate, and/or reverse the age-associated loss of muscle mass and strength (Devries, 2014; Forbes, 2012; Candow, 2012). A multiplex approach may eventually succeed in attenuating sarcopenia, whereas individual therapies cannot.

A first step in developing treatments is to create an animal model for pre-clinical analysis. The C57BL/6 mouse is a commonly used model of aging. In previous work we developed a frailty phenotype index for mice (Liu, 2014) based upon the Fried frailty phenotype (Fried, 2001). In a follow-up study we investigated the effect of a short-term voluntary aerobic exercise intervention and found that one month of wheel running was sufficient to reverse frailty in mice (Graber, 2014). The gold standard for sarcopenia intervention, however, is weight training not aerobic training, and we thus next produced and validated a mouse model of human weight training (Chapter 4).

The overall purpose of this thesis was to first investigate, characterize, and describe the neuromuscular healthspan of this mouse model; subsequently

creating a treatment model for sarcopenia. The first two research chapters (2 and 3) investigate the neuromuscular healthspan and the age-related decline of contractile parameters in the C57BL/6 mouse. Because the only consensus treatment for sarcopenia is resistance exercise, in Chapter 4 a mouse model of voluntary resistance training was designed and validated. The protocol was designed using human principles of weight training (Garber, 2011) and assessed with a comprehensive battery of outcome measurements. The outcomes were selected to test whether the mice had the same type of adaptations as would be observed in humans undergoing a similar training intervention (Kraemer, 2000). Finally, In Chapter 5, the resistance training protocol was applied to a cohort of aged mice to test the if signs of anabolic resistance would be detectable.

Prelude

The second chapter consists of the "*C57BL/6 Neuromuscular Healthspan Scoring System"*. Because there is a vast amount of individual variability in outcome measurements commonly used for function in aging studies (rotarod, grip test, physiology), the purpose of this study was to create a system that would serve to reduce the effect of this variability in order to increase the ability to detect changes resulting from interventions. Therefore, this study describes the C57BL/6 adult neuromuscular healthspan (5-32 months of age) using the performance measurements of rotarod and grip test and *in vitro* contractile physiology to measure the peak tetanic force of the EDL. These scores were combined in a mathematical construct (using multiple regression predicted scores and the means of the cohorts) that was able to greatly improve the statistical power of the individual outcome measures.

Because peak tetanic force is static, but animals move in life using power, in chapter 3, "*C57BL/6 Lifespan Study: Age-Related Declines in Muscle Power Production and Contractile Velocity"*, contractile velocity and power output over the lifespan of the mouse was characterized in both the soleus and EDL. Power is a critical determinant of functional ability and declines with age at a more rapid rate than force alone. Not only was a decline in function found, but that there was also a load-modulated age effect (the mice demonstrated increased age-related

dysfunction at measurements taken at higher percentages of maximum force). This finding has possible translational importance because power and velocity training may be one way to increase functional ability and to prevent disability in seniors. The more difficult tasks such as opening a sealed jar, getting up from a chair or preventing a fall require power production at the upper levels of maximum force. Therefore, since this part of the power curves declines more rapidly with age, we need to investigate interventions to help alleviate that loss. That could ultimately improve quality of life and extend independent years in the older population.

To date, strength training has been the gold standard to combat sarcopenia (with the goal of increasing strength and building muscle mass). Because there is not a mouse model that truly mimics human weightlifting as it would be performed in the gym, following the observations from the prior two studies, in the fourth chapter, "*Voluntary Resistance Training in Adult Mice"***,** a novel resistance training protocol using custom equipment (a powered running wheel and weight harness) and utilizing human weight training principles was developed and validated in an initial study using adult mice (12 months old at endpoint). One main question was, "Would training increase power and velocity, as well as functional performance and strength?" Using a carefully selected battery of outcome measures designed to test whether or not changes in function, physiology and morphology occurred as would be expected after following a

resistance training regimen, the protocol was determined to be a valid mimic of human weight lifting or resistance training.

Chapter 5, "*Voluntary Resistance Training in Elderly Mice and Signs of Anabolic Resistance"*, investigated the efficacy of the training protocol described in Chapter 4 to improve outcomes in an elderly population (28 months at the endpoint). While some of the same measurements improved as in the younger mice, others did not, or to a lesser extent. Thus, there was some evidence of anabolic resistance in the older mice.

Chapter 1 contains background information that is pertinent to the four main chapters, but that is not absolutely needed for understanding the individual research chapters. Each research chapter contains its own introduction explaining needed background and has a discussion explaining the context of the research to existing literature.

In summary, this is the story of age-related neuromuscular decline in performance and physiological function in that can be partially rescued through exercise. The effect of exercise was modulated by the age of the animal. Future work will investigate the genetic basis of the responder/non-responder differences, and adding synergistic interventions to complement exercise training

(nutritional supplementation of branched chain amino acids, for example) to improve exercise response in non-responders and older mice.

Chapter 1 Background Information

A. Aging Demographics

A rapidly aging population has caused a world-wide demographic change that presents multiple challenges for the socio-economic sustainability of the medical establishment. The so-called "greying of the population" stands to test the resolve and solvency of the healthcare system and governmental/societal resources. In 1900, the average age for survival was 47[7] and 4% of the population was over the age of 65 (3.1 million people). By the middle of the 20th century, median survival had grown to 68 and the number of people over the age of 65 in the United States was 12.3 million or about 8% of the overall population.

In 2007 the life expectancy at birth was 77.9 years[8]. The estimated population of the United States, as of 02/20/12, is 313,052,188 [2]. The projected population in 2030 is 373,504,000[9]. However, between 2010 and 2030 the percentage of traditional working age Americans (18-64 years of age) will decline from 63% to 57% while a concurrent increase in the number of Americans over the age of 65 increasing from 13-19%. Furthermore, the percentage of Americans over the age of 85 will increase by 25% and the percentage of centurions will double. Projections for 2030 do not include any radical technological biomedical advances that could even further increase lifespan; only actuarial data

[7] http://www.cdc.gov/nchs/data/nvsr/nvsr59/nvsr5909.pdf
[8] http://www.census.gov/
[9] http://www.census.gov/population/www/projections/summarytables.html

projections. The actual estimated (1900-2008) and projected (2010-2050) number of older Americans is displayed in Figure 1[10].

What are the implications of the aging of society? Beyond the obvious problems such as an actual net percentage loss in the number of working age tax paying citizens (which will result in a crippled ability to sustain Medicare and social security in the current tax/benefit structure), there are a host of quality of life issues to consider. Long-term health care facilities such as nursing homes are incredibly expensive**.** One hope is that along with the increasing aged population will come advances in biomedical technology that will allow a compression of mortality—people will not only live longer, but they will stay healthier longer and the end-of-life time of disability, loss of independence and sickness will be shorter. Interventions at the social level will also be vastly important—programs such as the non-profit Meals on Wheels that delivers nutritious food to homebound elderly clients (and has seen some erosion of funding in recent years)[11]. To be able to allow more of the elderly to maintain their independence longer by preventing or stalling the onset of sarcopenia and frailty will be critical to successfully managing this transitional period in which the 65+ elderly demographic is expected to become 25% or more of the total population by 2050.

[10] http://www.agingstats.gov/Main_Site/Data/2010_Documents/images/Indicator-1_Num_of_OA_left.gif

[11] http://meals-on-wheels.com/326

B. Sarcopenia

Sarcopenia, the age-related loss of skeletal muscle mass and strength, will become a vastly important condition as more people live longer and quality of life (healthspan) becomes more important than lifespan extension. Muscle mass typically peaks by the mid-20s-30s and then declines steadily after the mid-40s (Jansen, 2002). By the age of 80, people have lost about 40% of their peak muscle mass (Deschenes, 2004). The most serious cases of sarcopenia affect about 8% of older adults and are severe enough to cause functional disability (Jansen, 2002), such as the inability to perform necessary daily tasks such as dressing, bathing, cooking or even walking without aid.

Sarcopenia can ultimately cause the elderly to lose their independence and contribute to the onset of frailty—costing billions of dollars per year (direct cost of sarcopenia was $18.5 billion in 2002) (Jannsen, 2004). It is in the best interest of society, both financially and as a quality of life issue, to find successful interventions for this muscle wasting condition. The development of animal model systems that have the ability to assess neuromuscular healthspan is critical to monitor potential interventions for sarcopenia and frailty prevention.

A common definition of sarcopenia is an age-related decline in strength and muscle mass (Nair, 2005). Frailty is a little more difficult to define. One idea is that frailty is the loss of ability of the body to thrive and/or maintain homeostasis,

especially after illness or injury (Cooper, 2012). One recent clinical definition of frailty that has been developed for humans is having 3 of the 5 determinants (unexplained weight loss of >10 pounds, low grip strength, low energy level, slow gait speed and low activity) qualifies one as being frail (2 of 5 is equivalent to “pre-frailty”) (Fried, 2001). Neuromuscular dysfunction and sarcopenia play a large role in the clinical manifestation of frailty (Fairhall, 2011, Cooper, 2012).

Much research has delved into the etiology of sarcopenia, and the condition has a multi-factorial basis, some examples being: denervation, disuse atrophy, lowering of muscle quality (at multiple levels: from connective tissue dysfunction to fat intrusion to contractile protein damage), impaired signaling pathways regulating protein synthesis and catabolism, and lowered response to anabolic stimuli (Ryall, 2008; Thompson, 2009; Narici, 2010; Fielding, 2011).

Studies show that fewer than 2% of middle-aged and elderly people regularly (more often than once per week) participate in weight-bearing exercise (Jansen, 2002). Though only about a five percent loss of fibers is seen by age 50 (with a rapid increase in percentage lost after age 60) (Deschenes, 2004); the more muscle built in youth and maintained throughout life, the more mass there is to be lost as sarcopenia begins. Even master athlete weight lifters and marathon runners (defined as elderly people who have trained for decades) decline in

performance an average of 50% between the age of 40 and 80 (Faulkner, 2007). Sarcopenia can be treated, but not cured.

Many studies have shown that resistance exercise is successful in increasing muscle mass and strength in the elderly, with the additional side effects of bone density increase, body composition improvements, and better metabolic function (Melov, 2007; Fiaterone, 1990; Frontera, 1988; Aagard, 2007; Phillips 2007, Kalapotharakas, 2005). Beyond the issue of the loss of mass, muscle quality (contractile power of individual fibers) declines from a weakening of the contractile strength of the actin/myosin network—this has been shown to be reversible to a degree with exercise (Snow, 2004). Functional strength and quality of daily life can be improved and/or maintained with exercise (Phillips, 2007; Kalapotharakas, 2005). Frailty can be avoided and independence maintained (Borst, 2004).

Yet another consequence of reduced strength is decreased mobility, and by extension, independence (Jansen, 2002; Kauffman, 2007). Often the energy and strength necessary to perform routine daily tasks is lacking, leading to removal from independent living and the resulting transition into managed care. Overall, the loss of muscle mass in the later stages of life contributes to a lower quality of life (Kaufmann, 2007).

Beyond quality of life issues, there are legitimate health concerns involved with the decrease of muscle mass, strength and power (Phillips, 2007). For example, skeletal muscle is the single largest metabolic engine burning vast quantities of glucose (and by default fats), outside of the liver (Phillips, 2007)). The basal metabolic rate is the amount of energy burned by an individual at rest. A person with more muscle burns more energy, even when not exercising than a person with less muscle, because fat cells are mostly metabolically inert in comparison to muscle cells (Whitney, 2005). Therefore, loss of muscle mass lends to a decrease in metabolic rate and, without strict caloric restriction, results in an increase of stored body fat. An increase in body fat percentage and BMI (Body Mass Index) has been correlated with an increased risk for metabolic syndrome and all of its attendant consequences (increased risk of cardio-pulmonary disease, diabetes, etc.) (Whitney, 2005). Many other effects, such as increased pressure on overtaxed joints and self-esteem/image issues also result from fat gain (Whitney, 2005).

Another example of sarcopenia related health issues is falls. With age, there is also decrease in the effectiveness of the inner ear balancing mechanism, the vestibular system (Kaufmann, 2007). When stimulated, the hair cells that line the inner ear release neurotransmitters to the attendant axons, or nerve cells. They are surrounded by a gelatinous material (which constantly pulls the hairs in

response to gravity, thus causing a sense of up and down) that is heavier than the endolymph filling the semicircular canal, the utricle and saccule. These three parts of the area of the inner ear are separated from the cochlea by the “oval window”, and are responsible for balance and spatial orientation. These hairs become less sensitive to the changing motion of the inner ear fluid (endolymph) as they become senescent; and, thus, the body has a harder time orienting itself in space (Campbell, 2005). This vestibular system decline, as well some prescribed medications and blood pressure problems, can cause a decrease in balance in the elderly. Along with a loss of strength and power (power being the application of force over time) due to sarcopenia, the tendons connecting the muscle to the bone decrease in elastic modulus and stiffness, resulting in a reduced and slowed transmission of force (McCarthy, 2014; LaCroix, 2013, Narici, 2008). The neural connection from the brain to the muscle cells declines in effectiveness from an age-associated reduction in nerve conduction velocity due to age-associated demyelination of the peripheral nerve (Manini, 2013). When an individual begins to lose balance, they attempt to regain it by increasing force, for example, in one leg. The little used motor program for “arrest fall!” signals the remaining motor neurons (after age-related denervation) to send an action potential to stimulate motor units made up of smaller numbers of muscles fibers than in younger people. These smaller motor units are more type 1 than type 2 (better at powerful movements) and the available fibers are also smaller and of lesser quality (weaker per unit cross-sectional area) than what would be

available in a young person attempting to stop a fall. After the fibers in the motor unit are activated, there is a further delay in response at the joint because the tendon is not taut and the muscle force is delayed in reaching the needed pivot point (Phillips, 2007). By this time, the person has fallen and perhaps risked serious injury. Falls that result in severe injury form the leading edge of a downward spiral of failing health leading to mortality (Borst, 2004).

Attempting to find treatments to combat sarcopenia has been less than successful. Many therapies have shown some promise on a limited scale (nutritional, pharmacological and ergogenic) but no single therapy has proven overwhelmingly effective (Candow, 2011). To date, the best and most accepted therapy for sarcopenia is resistance exercise, weight training in particular (Fiaterone, 1990; Capodaglio, 2007). In the future, a multi-prong approach utilizing a number of different therapeutic strategies in synergy with resistance exercise will prove to be the answer to address the attenuation of sarcopenia.

Prevalence of Sarcopenia

Progressive loss of muscle mass and strength with age may be inescapable with current medical technology. Whether this loss is termed sarcopenia or not is at the level of semantics, but does the condition impact functional ability? There has been an international effort to find a clinical definition of sarcopenia. One definition is that after seeing manifestations of phenotypical symptoms

(weakness, difficulty in performing activities of daily living, etc.) that the patient be evaluated via DEXA (dual energy x-ray absorptiometry). Individuals who present at less than the 20th percentile of lean appendicular mass of healthy young adults and who have functional impairment can be diagnosed with sarcopenia (Fielding, 2011).

C. Effects of Aging on Muscle

Age-related changes to muscle cells are many and varied and occur on a variety of levels. Sarcopenia is the result of muscle atrophy (often related to disuse), denervation and loss of muscle fibers as well as a reduction in muscle quality (defined as the ability of a given volume of muscle to produce force or power) (Thompson, 2006). With age, there is a decrease in muscle cross sectional size, and in the overall number of muscle fibers, an increase in fat deposits and connective tissue within the cells, and disruption of many important signaling pathways (Porter, 1995).

Lifestyle changes in older adults, in particularly lower activity levels, contribute to decline in functional ability (Marini, 2008). Many of the support systems that help to maintain muscle function are negatively affected by age. For example, the endocrine system produces fewer neurotropic growth factors→production of growth hormone declines, which in turns causes a reduction in production of IGF-1, the major growth factor affecting skeletal muscle. Less anabolic hormone production (i.e. free testosterone levels decline) and a corresponding increase in catabolic hormones, e.g. cortisol, results in decreased anabolic capability (Stenholm, 2010). Excess inflammation and the resulting catabolic response have been attributed as one of the many causes of sarcopenia (Deschenes, 2004). Neuromuscular decline in the peripheral nervous system could result from decreased activity (Deschenes, 1994). The cardiovascular system declines in

ability—from reduced cardiac output to a lessening of capillary connection to muscle fibers--reducing muscle function by limiting nutrient and oxygen availability to cells (Guarner-Lans, 2011). Nutrient absorption is also negatively affected by age, resulting in a need for increasing levels of micro and macro-nutrients in the diet to provide need building blocks for muscles (protein intake must be increased in the elderly for example) (Koopman, 2009; Breen, 2011). Reduced insulin response in older adults causes a lessened ability to uptake glucose. Neurological changes also contribute to the overall decline of the system as some α-motor neurons begin to decay and denervate muscle cells—ultimately resulting in cell atrophy and apoptosis.

Denervation consists of the loss of motor units and the subsequent atrophy and apoptosis (cell death) of the muscle fibers associated with the motor unit (Faulkner, 2007). This process occurs disproportionately in fast twitch muscle cells (cell designed for rapid contraction, power and anaerobic metabolism). The fiber, after losing its neural connection, is either connected to a neighboring (often slow-twitch) motor unit network through axonal sprouting (which then converts the fiber to a slow twitch), or it atrophies and dies (Koopman, 2009; Breen, 2011, Ryall, 2008). Denervation, and subsequent reinnervation (whether by another branch from the denervating neuron, or with a new branch from a neighboring neuron), are balanced in youth, but become unbalanced in favor of denervation in older adults, resulting a net loss of fibers. Between age 50 and 80,

approximately 50% of muscle cells are lost (Faulkner, 2007). Much remains to be investigated concerning this process. IGF-1 (insulin-like growth factor 1) upregulation, for example, was found to prevent the loss of fast twitch motor units in rats (Messi, 2003).

Other reasons for decreasing muscle mass are related to a decrease in the size of the existing muscle fibers. This is often measured using the cross sectional area of the fibers. Some reasons given for the age-related decrease in fiber size include: signaling pathways disruption resulting in reduced response to anabolic stimuli, increased expression of catabolic hormones, lessened production of hormones (growth, androgenic and anabolic), oxidative stress damage to muscle cell organelles and structure (perhaps also contributing to apoptosis), and disuse atrophy (Ryall, 2008; Faulkner, 2007).

Age-related changes in the endocrine system possibly contribute to anabolic resistance. Anabolic hormones are critical to muscle building hypertrophy (Gao, 2007). The decline in production of testosterone, and its precursors, such as DHEA (dehydroepiandrosterone), is termed andropause, and occurs in both males and females (documented as being up to 64% and 28%, respectively for testosterone) (Van der Beld, 2000). Testosterone production in men begins to decline in the mid-to late-20's.

At the tissue level, reduced stem cell proliferative capacity provides a declining ability of elderly muscle tissue to repair damage by reducing the ability of satellite cells to self-replicate and to differentiate into nuclei that fuse to muscle fibers (Jones, 2011). The muscle has intrusion of fat and connective tissue where contractile element used to exist, reducing muscle quality (Addison, 2014). Both innervation and blood supply control are reduced or less effective, further causing declining function (Narici, 2008; Payne, 2004, Payne, 2006; Procter, 2006). Paracrine emission of cyto/myokines by aging muscle change to negatively affect growth and repair ability, and enhance atrophy (Pratesi, 2013). Connective tissue required to transmit force from muscles also have age-related changes, such as reduced tendon elastic modulus, that negatively affect movement (Onambele, 2006). Joints also suffer deterioration and loss of function (Lanza, 2003). In essence, the whole neuro-skeletal-muscle system declines in functional ability with age (Faulkner, 2007; Porter, 1995).

At the cellular level, many changes occur with age. Some changes happen because of cellular level or biochemical processes. A good example of this is damage from oxidative stress. If a muscle, a post-mitotic tissue, is continually bombarded with reactive oxygen species (byproducts of cellular respiration), eventually accumulated damage will occur beyond the capacity of the cell to repair. This results in a backlog of damaged proteins that may be dysfunctional or nonfunctional. Damage and/or misfolded proteins result in lessened enzymatic

capability and can affect varied cellular processes from glycolysis to force production. While many of these proteins are cleared out by the action of proteases (proteasome for example) or repaired by heat shock proteins, some may result in ultimately causing the demise of organelles or will cluster to form aggregates that resist the ability of the cell to recycle or repair. Repair and recycling mechanisms also decline over time, so a vicious cycle is set up. Mitochondria, for instance, may become dysfunctional over time (mitophagy clears out many but not all “broken” mitochondria) and the accumulation of nonfunctional organelles within the cell reduce volume available for contractile elements (hence a reduction in muscle quality).

Another problem is DNA damage (from ROS or spontaneous mutation over time) which can result in mitochondria dysfunction when mitochondrial DNA is damaged or even dysfunction nuclei, which can then have transcription problems. Ribosomal protein synthesis ability is also lessened over time, so translation of new proteins to replace damaged proteins becomes an issue. In general, it appears that there is reduced rate of protein synthesis seen with age (Breen, 2011); therefore maintaining the homeostasis of the cell by balancing production of new proteins with the catabolism of old proteins may become more difficult. Of course, ROS influenced post-translational modifications could be theorized to reduce function at many different levels.

Molecularly, the ability of the myofiber to function may also become impaired with age. For many of the aforementioned reasons, certain integral processes to muscle function might become compromised with age. Many examples abound. The cross-bridge cycle, which produces force in the muscle, relies upon numerous aspects of cell biology to function at an optimal level, and deleterious changes in membrane excitability and calcium handling contribute to age-related dysfunction in muscle (Payne, 2009).

The actions of the dihydropyridine receptor (DHPR) and the ryanodine receptor (RYR) in response to the depolarization of the action potential (AP) must make enough calcium available in sufficient amount to load onto and saturate troponin c. This causes a conformational change in the troponin complex that moves tropomyosin off of the myosin binding sites on the actin thin filament, thus allowing the myosin head to engage and strongly bind to the actin filament—initiating the cross-bridge cycle. DHPR are voltage-gated complexes that change conformation in response to the depolarization of the action potential and physically interact with RYR, causing the RYR to open their channels to release a flood of calcium from storage in the sarcoplasmic reticulum. A reduction in the density of DHPR with age could result in fewer RYR in contact with DHPR, thus resulting in a lessened ability for calcium from the sarcoplasmic reticulum to be released in response to a given AP (Delbono, 2011).

A reduced ability of motor neurons to provide acetylcholine (smaller and more disorganized endplates occur with age) results in weaker AP which can further contribute to calcium handling dysfunction (Tudorascu, 2014). ATP must be available in sufficient quantity to phosphorylate myosin and to activate the calcium ATPase pump to reuptake calcium into the sarcoplasmic reticulum during relaxation.

One very important additional alteration with age is the change in myosin heavy chain and myosin light chain (MLC) isoform distribution. Aging is associated with a reduction in MHC2 overall, and in the faster isoforms such MHC2b (See Chapter 2 results). These heavy chains cross-bridge faster than MHC1 and so can produce more power. Furthermore, MLC come in numerous isoforms (fast fibers have MLC1f and MLC3f) as well. These MLC isoforms also influence the speed of cross-bridging, MLC3f being the fastest. Age sees a reduction in MLC.3f content (See Chapter 3 results), thereby further slowing the type two fibers.

D. Aging, Muscles and Resistance Training

There is hope to attenuate much of age-related muscle dysfunction. Exercise provides nearly universal benefits to help recover function and to slow declining ability, regardless of the starting capacity of the individual. Even the frailest of the elderly benefit from increased activity (Fiatarone, 1990; Galvao, 2005).

Two types of training are often advocated: aerobic and resistance. Aerobic training can be further subdivided into weight bearing and non-weight bearing motifs. Examples of weight bearing aerobic exercise include walking or jogging. Non-weight bearing exercise might include stationary bike riding and swimming (although swimming is also mildly resistive in nature). Hybrids also are seen (partial weight bearing) such as water aerobics. The main benefits derived from aerobic-style exercise, as the name implies, are to aerobics systems such as the cardio-vascular system. A huge list of benefits to overall physiologic health occur with aerobic exercise including, but not limited to: improved body composition, fatty acid oxidation and insulin sensitivity; increased VO_{2max}; better fatigue resistance, increased bone mass at weight bearing areas, improvement in joint function and some limited hypertrophy of type one muscles (Allen, 2011). Many of these changes benefit the overall environment of the body and can help contribute to increased mobility. While a sedentary individual will see great improvement from any sort of increase in activity, aerobic included; to truly

improve muscle function and attenuate sarcopenia, resistance training is required.

Resistance training includes weight bearing calisthenics and weight lifting. Weight lifting is the gold standard in improving muscle function. Muscle hypertrophy resulting in increased muscle mass and strength is the main advantage that resistance training has over aerobic training. In addition, resistance training also provides many of the same type of benefits as derived from aerobic exercise, albeit at a lower level (Phillips, 2007). Improved fatty acid oxidation, body composition, insulin response, increased capillary density and so forth all derive from weight lifting as well. While these benefits may be received at a high level in aerobic style exercise, aerobic exercise does little to increase force production and muscle mass (it primarily acts upon type 1 muscle and to a limited extent upon type 2a). Since problems with type 2 muscles are generally the plague of the elderly (slow twitch muscle being generally more resistant to age-related dysfunction) (Thompson, 1994), resistance training is the best hope to improve the type 2 motor units. Much of the atrophy seen in the elderly may be disuse atrophy resulting from lifestyle changes and decades of a sedentary lifestyle (denervation of type two muscle may also have a disuse component. Therefore, to combat muscle loss that results in lower power output and movement dysfunction, a program of weight lifting is ideal.

Many of the adverse age-related changes to the muscle can be at least partially reversed by implementing a well-designed program exercising all of the major muscle groups using progressive resistance and sufficient intensity, duration and frequency (Chodco, 2009). While exercise response is blunted in the elderly in comparison to younger people (Hunter, 2004; Leiter, 2011), it still can cause increases in anabolic hormone, growth factor and paracrine/autocrine production, cause muscle cell remodeling and hypertrophy (clearing out old damaged proteins and adding new, fresh proteins and contractile elements), increase strength and power production thereby improving functional ability, an increased satellite cell pool with improved proliferative capacity and many other positive changes to both the muscle fiber, whole muscle, muscle environment and support systems (Hunter, 2004; Leiter, 2011). Of course, adequate nutrition is important as well—older people generally require more protein and nutrient signaling pathways are somewhat disrupted requiring a careful attention to nutrient intake (Koopman, 2009).

In conclusion, with respect to increasing mobility and to help restore functional ability in the elderly, any exercise is good exercise. The best prescription is for a combined approach, such outlined in the American College of Sports Medicine position statement on exercise and older individuals, with aerobic, resistance training and flexibility training all playing a part (ACSM, 2008). The greatest

benefit for improving skeletal muscle function itself comes from resistance exercise.

E. PI3k→Akt→mTORc1→p70S6K Signaling Cascade

One of the main pathway of protein synthesis associated with skeletal muscle hypertrophy begins with the activation of PI3k (phosphoinostisol-3 kinase) by

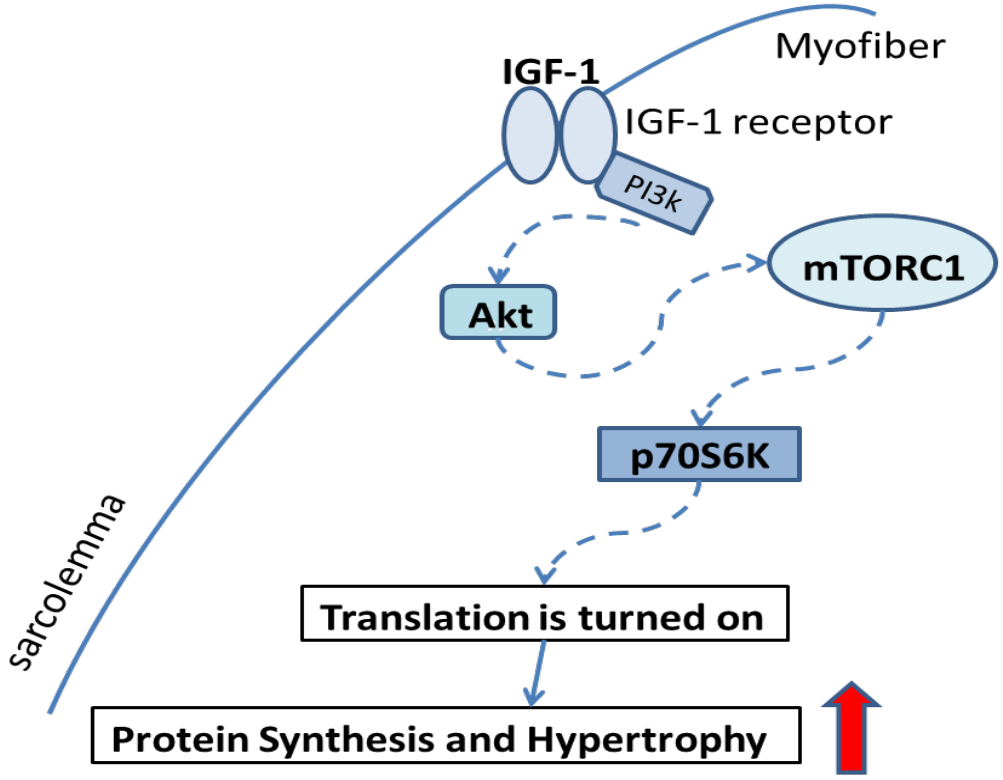

Figure 1 Simplified Growth Factor→PI3k→Akt→mTORC1→p70s6k Signaling Cascade. Akt is phosphorylated at the Threonine 308 site, then downstream mTORC1 is phosphorylated at the Serine 2448 site, and p70s6k is phosphorylated at Threonine 389 (one of many sites on p70S6K, but which is required for protein translation). p70S6K phosphorylates ribosomal protein S6 and eIF4B (eukaryotic translation initation factor 4B), afterwhich the ribosomal complex can form and translation of mRNAs can begin, ultimately resulting in protein synthesis.

growth factors which then eventually downstream turns on Akt by phosphorylation, which then goes through the mammalian target of rapamycin complex 1 (mTORC1) to turn on translation, in part by phosphorylating p70S6K (Glass, 2010). There are a number of important regulators upstream of mTORC1. Important external signaling mechanisms are growth factors (insulin-like growth factor 1—IGF-1—for example), nutrient signaling (e.g. branched chain amino acids, leucine in particular, upregulated mTOR), and negative signaling molecules such as myostatin.

F. Etiology of Anabolic Resistance

Age-Associated Systemic Physiological Changes

Hormonal

Anabolic hormone production declines with age and may be one root case of sarcopenia out of many (Sipila, 2013, Valenti, 2010). Testosterone is a highly anabolic hormone produced by both men and women, though to a much larger level in men. Testosterone production in men begins to decline in the mid-to late-20's and then continues to declines, on average, 1% per year after age 40 in men (Gray, 1991). Testosterone is only bioactive in its free state, not when bound to SHBG—sex hormone-binding globulin. SHBG increases more than 2-fold in quantity with age (Van den Beld, 2000). The decline in testosterone production, in both males and females, has been documented as being up to 64% and 28%, respectively (Van der Beld, 2000). There has been research into utilizing anabolic steroids and/or supplemental testosterone (or precursors) in aging men to restore vitality and muscle mass. Anabolic steroids, and exogenous testosterone, definitively work to increase lean muscle mass (Myhall, 1998). A meta-analysis of testosterone therapy showed a clear benefit of increasing strength (Ottenbacher, 2006). One major confounder is that neither exercise nor diet was prescribed or controlled for in most of the studies. This would limit the potential for therapeutic benefit to be gained by improving the anabolic environment (with anabolic hormonal supplementation) but not providing a potent

anabolic stimulus (resistance training, and protein ingestion). Perhaps combining resistance training with anabolic supplementation could be a winning strategy. In addition, most of these studies involve restoring hypogonadic males to age-normal levels of testosterone, restoring to high-normal levels of young men or to slightly super-physiological levels would have a greater impact (Deschenes, 2004). There has been funding shortage for investigating hormone replacement therapy (Lynch, 2004a) and its association with so-called aging-clinics has lowered respect for the field. Most studies now conclude that, while effective, dosage levels and side effects incurred, as well as adverse reactions seen in limited form, require much more study to be made in order to formulate effective clinical strategies for administration of steroids as an anti-sarcopenia strategy.

Somatopause (similar to andropause- the reduction of testosterone) is the lowering of blood borne levels of growth hormone (somatotropin). Generally men begin to have a gradual decline in GH levels starting in the early 30's that progresses (Sattler, 2013). GH has effects that are expressed downstream through the liver produced IGF-1 pathway but it also affects muscle in a direct fashion. GH has a connection to numerous important metabolic functions including: mitochondrial efficiency, improving overall body composition by increasing fat metabolism and increasing resistance to fatigue (Ryall, 2008; Sattler, 2013). Growth hormone administration in great excess of therapeutic levels leads to fluid retention, growth of soft tissue such as the cartilage in the

nose and jaw, and organ growth. To date, it has not been proven to be an effective strategy to use GH to stimulate muscle mass increase (Lynch, 2004b) or strength (Borst, 2004), though GH has been shown to improve body composition and general well-being (Sattler, 2014).

Insulin and insulin-like growth factors (IGF-1 and 2) are also powerful anabolic agents. Much IGF is produced in the liver and is controlled by growth hormone. Other IGF-1 is produced and used locally inside the muscles. There are at least two splice variants produced in the muscle itself, IGF-1Eb (sometimes referred to as MGF, or mechano growth factor) and is released in response to physical stimulation (Ryhall, 2008). The other splice variant (IGF-IEa) is nearly identical to the liver-produced splice variant. Active IGF-1, after post-translational modification, is identical, no matter which splicing it originated from. However, the regulation of IGF-1 by binding proteins and regulation of intra-muscular production has not been fully elucidated. Some forms of IGF-1 binding proteins are more prevalent in older individuals than in younger (Ryhall, 2008). Some evidence exists that IGF-1 plays an insignificant role in contraction-mediated protein synthesis (Hornberger, 2011; You, 2014). However, IGF is important in regulating apoptosis and denervation (Messi, 2003), as well as in satellite cell activation and differentiation. More research is needed to examine the role of IGF-1 in anabolic resistance.

Thyroid hormone production also changes with age (Aggarwal, 2013). Thyroid hormones are responsible for many metabolic functions, many of which either directly or indirectly affect skeletal muscles. Hypo-thyroid predicates changes in muscle fiber phenotype (from fast twitch to slow twitch forms) and in control of calcium ion transfer across the sarcoplasmic reticulum (Ryhall, 2008).

Thus, the age-altered hormone levels may play a role in the systemic environment contributing to anabolic resistance. In addition to declining hormone levels, another possibly potent mediator of anabolic resistance at the systemic-level is global inflammation. Some studies have linked age-associated global inflammation to reduced capacity to respond to exercise (Merritt, 2013, Lawler, 2011). Much of the repair mechanisms in muscle that would be activated after a vigorous exercise routine rely upon inflammatory signaling to activate response. Thus, it is conceivable that a high level of background noise (global elevation of inflammatory signaling) may obscure the normal repair signaling response, thus limiting adaptation.

Age-Associated Changes to the Cellular Environment

Satellite Cells

Satellite cell are muscle stem cells that perform a critical function in repair of damaged muscle fibers, and have long been thought to contribute to hypertrophy by fusing to existing muscle fibers. There is a reduced capacity for satellite cell functioning in older subject (Conboy, 2005; Gopinath, 2008). Despite some recent data showing that satellite cell function may not necessary for hypertrophy, at least as far as maintaining the myonuclear domain (Jackson, 2012), there is no question that older subjects have a decline in proliferative/differential capacity which certainly hinder injury recovery, and possibly exercise response. More research is needed in this area.

Impaired Protein Synthesis Pathways

Anabolic signaling pathways are stimulated by growth factor (IGF-1 for example), nutrients (for example the branch chain amino acids leucine stimulates the mTor pathway) but may not as responsive in the elderly as in younger individuals (Burd, 2013). Protein synthesis rates also decline with age in response to nutrient signaling (Walker, 2011). There is some evidence that amino acid transporters play a role in the age-related reduced anabolic response to protein feeding (Dickinson, 2013). Evidence has recently been discovered that mechanical stimulation increases protein translation via an Akt independent mTOR signaling mechanism involving diaglycerol kinase (Hornberger, 2011). More research is

needed to investigate whether this mechanical stimulation anabolic signal is blunted in the elderly.

G. Measuring Skeletal Muscle Contractile Physiology: The Basics

This section is not meant by any means to be an exhaustive review of muscle physiology, it is simply a brief overview of the cascade of events that occur from the moment a motor neuron fires down to the eventual application of force across a joint—in the context of the difference between measuring contractile parameters *in vivo* or *in vitro*. Skeletal muscle is voluntary muscle (i.e. can be controlled with conscious thought). Therefore, once the decision has been made by the central nervous system to use a muscle and the signal from the cerebral cortex is conveyed and refined through the motor cortex and sent out to the spinal cord, an action potential is generated at the cell body of an alpha motor neuron and conveyed down the axon to eventually reach each neuromuscular junction associated with that motor unit. This action potential causes a release of acetylcholine, stored in synaptic vesicles in the nerve ending. The acetylcholine then diffuses across the synaptic cleft to the end plate (invaginated sarcolemma side of the neuromuscular junction) and attaches to the acetylcholine receptor, opening its ion channel, the resulting influx of ions then opens associated voltage-gated sodium/potassium ion channels initiating a local depolarization in the sarcolemma. This local depolarization becomes an action potential and travels along the sarcolemma and deep into the cell via the transverse tubules. The action potential then stimulates the calcium handling proteins initiating excitation/contraction coupling and force generation (discussed in depth below).

The depolarization process is where the essential difference between the isolated preparation and the *in vivo* state begins.

The absolute main difference between muscle contraction *in vivo* and the isolated muscle prep is that *in vivo* the muscle is innervated and in the isolated muscle prep *(in vitro*), for all intents and purposes, there is no nerve involvement in the generation of the action potential. Therefore, both the method and process of depolarization is different. In the *in vivo* model, the action potentials flowing down the motor neuron to the neuromuscular junction (generally in the center of the muscle cell) must result in releasing enough acetylcholine (2 molecules for each receptor) into the synaptic cleft (for the underlying acetylcholine receptors (AChR) to allow a small amount of potassium out and larger amount of sodium (both traveling down their concentration gradient) into the cell to cause a local depolarization (known as the end plate potential). This local depolarization causes nearby voltage gated sodium channels to open causing a flood of sodium into the cell which amplifies the depolarization and sends it propagating outwards as an action potential (AP). Acetylcholine esterase (AChE) is an enzyme located in the basement membrane that captures some acetylcholine (ACh) on the way down to the AChR, but since they are far less abundant than the AChR, most of the ACh gets through. After the AChR pores open and the ACh molecules are released, the AChE captures the diffusing ACh and hydrolyzes them, ending the synaptic transmission.

The AP then propagates through the rest of the myofiber by traveling along the membrane and into the interior of the cell via the transverse tubules (invaginations of the sarcolemma continuous with the exterior of the cell). In the *in vitro* prep, the muscle sits a temperature controlled bath containing a solution such as Krebs buffer (containing electrolytes like sodium, potassium and magnesium at physiological concentrations of interstitial fluid along with a pH buffer and sugar) in between two electrodes with the tendons attached to force transducer/length controller or a static point via suture line tied at the myotendinous junction. The electrodes are stimulated to emit enough electricity at a given amperage and voltage at a certain pulse of a particular amplitude, length and frequency to maximally depolarize the entire muscle. Thus, ACh plays no part in the story for membrane depolarization in the in vitro prep—the voltage gated channels all open due to the external electrically impulses being applied. At this point, the *in vivo* and *in vitro* situations begin to become similar.

The AP traveling down the transverse tubule and depolarizing the local environment acts upon a voltage sensitive ion channel, the dihydropyridine receptor (DHPR). In response to the depolarization, the DHP is activated and changes conformation. Extending out of the sarcoplasmic reticulum and into the sarcoplasmic space between the SR and transverse tubule is a massive protein complex, the ryanodine receptor (RYR)--a type of calcium channel. There is a tetrad of DHPRs sitting directly over every other RYR and connected

mechanically to the RYR. One theory of how the RYR is activated involves the physical interaction between DHP and RYR. The conformational change of the DHPR activates the ryanodine receptor thereby opening a calcium channel (Felder, 2002). Calcium floods down its concentration gradient (thousands of times higher in the sarcoplasmic reticulum in comparison to the cytosol) from the calcium stored within the lateral sacs of the sarcoplasmic reticulum and out through the pores of the RYR into the cytosol of the muscle fiber. The calcium acts to both as a positive feedback loop to induce more calcium release in the short term (Ca^{2+} induced Ca^{2+} release) and diffuses to the contractile elements of the cell where it binds to troponin C initiating a conformational shifting of the troponin complex--thus allowing tropomyosin to move and the myosin binding sites on the actin thin filament to become exposed allowing cross-bridge cycling to occur. This ultimately results in force production and the process is known as excitation-contraction coupling (Delhunty, 2006).

Therefore, in essence, everything is the same in both preparations as far as excitation-contraction coupling goes. What is different is how the excitation portion is initiated. The process of membrane depolarization is marked different in the two systems as is noted above. Interestingly, this causes overall differences in a physiologic level above the individual cell. The entire muscle in vivo consists of numerous myofibers of whatever various types (I, IIa, IIb, IIx and hybrids) organized into motor units each innervated by an individual α-motor

neuron of a particular type (i.e. only type I motor neurons innervate type I fibers). When a muscle begins to contract, fibers are recruited to perform the task required based upon the size principle: smaller fibers and motor units are recruited first with progressively larger units held in reserve unless needed. This has the practical application of type I motor units being recruited first for “light weight” tasks (which the fatigue resistant fibers can perform for long periods) with multiple units being rotated with time to maintain force of necessary. If the force requirement for the task is great enough the motor units for larger, more powerful (and more fatigueable) type II muscles will be brought to bear. Thus, the innervation serves a mechanism to regulate gain in the system and modulate energy expenditure to produce only as much force as needed. In the situation of the *in vitro* preparation, the influence of the motor units have been replaced with an “all or nothing” firing pattern—the entire muscle contracts at once, maximally, because every fiber of every motor unit is depolarized at the same time by the electrical impulse stimulation. This has implications in the application of electrical pulses during the *in vitro* prep to stimulate the increased amount of calcium present in the cytosol of the cell necessary to produce tetanus rather than just a single twitch.

Consciously inducing a single twitch in a muscle, *in vivo,* is a difficult concept. In vitro, a single pulse of energy, maximally potentiated to fully depolarize the muscles in the prep, will produce a twitch. This twitch represents the ability of the

muscle to release calcium as well as the ability of troponin to bind it. In an EDL of a mouse, for example, a typical system might stimulate the muscle with 1 amp at 30 volts in a 300 ml bath with a single pulse of 200-microsecond duration. This pulse will depolarize the muscle cells, causing the DPHR to act upon the RYR and release calcium sufficient for the muscle to produce a twitch force of perhaps to a maximum of around 90 milliNewtons (mN). The muscle is set to its optimal length (the length at which the highest peak twitch force is produced or P_t). If, instead of a single pulse, a charge train of ten hertz frequency of 1 second duration is sent through the electrodes, ten individual twitches will be produced. This is because the frequency of the pulses are not high enough to produce a greater outflow of calcium from the RYR than can be pumped back into the SR by the SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase—a pump that uses ATP to actively push calcium into the SR form the cytosol against its concentration gradient and which is aided by calsequestrin, a calcium binding protein in the SR that helps to store the calcium) or pumped out of the cytoplasm by other calcium pumps. If, however, the frequency is increased to around 80 hertz the amount of calcium released is greater than what can be pumped back in the given time period and the subsequent stimulations occur before complete relaxation of the muscle is achieved and a fused tetanus occurs—resulting in a far greater production of force over a longer period of time (perhaps to a maximum of around 300 mN). With increasing hertz, the muscle will produce increasing force until a plateau is reached where total calcium saturation of the

troponin molecules is achieved. Of course, this plateau is individual to the muscle and depends upon many factors including the size of the fibers, the temperature of the bath and so on. It is often achieved between 120 and 180 hertz in the situation described above and the force generated varies individually as well.

The great advantage of the *in vitro* methodology resides in the ability to remove the outside influences of the physiological system and to simplify the number of confounders inherent to force production. There is no aspect of the environment that is not under the control of the researcher (temperature, ionic conditions, hormones, nutrient supply, etc.) and the concept of motor unit and neuron innervation does not come into play. The researcher easily controls the depolarization and the calcium handling of the muscles through the electrical stimulator. However, this advantage could also be looked as a disadvantage when results of experiments are extrapolated to *in vivo* systems where the complex interplay of numerous factors exterior to the muscle cells themselves are important regulators of force production. General Muscle Physiology background in this section referenced from Mackintosh, Gardiner and McComas, 2006.

Chapter 2

***This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Journal Gerontology A Biological Sciences Medical Sciences* following *peer review. The definitive publisher-authenticated version* Ted G. Graber, Lisa Ferguson-Stegall, Jong-Hee Kim, and LaDora V. Thompson. C57BL/6 Neuromuscular Healthspan Scoring System *J Gerontol A Biol Sci Med Sci (2013) 68 (11): 1326-1336 is available online at:* doi:10.1093/gerona/glt032.**

C57BL/6 Neuromuscular Healthspan Scoring System

Ted G Graber, Lisa Ferguson-Stegall, Jong-Hee Kim, LaDora V Thompson

University of Minnesota Medical School
Department of Physical Medicine and Rehabilitation
Minneapolis, Minnesota 55455

Ted G. Graber: primary study designer; performed all experimental data collection, data analysis/interpretation; primary writer of manuscript

Lisa Ferguson-Stegall: provided assistance with tissue collection, scientific review of manuscript

Jong-Hee Kim: scientific review of manuscript

LaDora V Thompson: responsible for all aspects of study, guarantor of the study, provided critical assistance in data interpretation and writing of manuscript, funding; email: thomp067@umn.edu.

Corresponding Author:

LaDora V Thompson, thomp067@umn.edu, 612-626-5271 (office), 612-624-2436 (fax)

Abstract:

Developing a scoring system based on physiological and functional measurements is critical to test the efficacy of potential interventions for sarcopenia and frailty in aging animal models, therefore, the aim of this study was to develop a neuromuscular healthspan scoring system (NMHSS). We examined three ages of the male C57BL/6 mouse: adults (6-7 months old, 100% survival), old (23-25 months old, 75% survival) and elderly (28+ months old, ≤50% survival) mice --as well as mice along this age continuum. Functional performance (as determined by the Rota-Rod and inverted-cling grip test) and *in vitro* muscle contractility were the determinants. A raw score was derived for each determinant and the NMHSS was then derived as the sum of the individual determinant scores. In comparison to individual determinants, the NMHSS reduced the effect of individual variability within age groups, thus potentially providing an enhanced ability to detect treatment effects in future studies.

Introduction:

The rapidly aging population has caused a worldwide demographic shift that presents multiple challenges for the socio-economic sustainability of the medical establishment (Herrell, 1980; Peterson, 1999). Sarcopenia, the age-related loss of skeletal muscle mass and strength, has become a more prevalent condition as more people live longer. Furthermore, quality of life during aging, or healthspan, becomes an important concern. Sarcopenia can ultimately cause the elderly to lose their independence and contribute to the onset of frailty—costing billions of dollars per year (direct cost of sarcopenia was estimated at $18.5 billion in 2002) (Janssen, 2002; Janssen, 2004). It is in the best interest of society, both from a financial standpoint and as a quality of life issue, to find successful interventions for this age-associated muscle-wasting condition.

Biogerontology research has traditionally focused on mechanisms to extend lifespan (Sierra, 2009). Recently, however, there has been a paradigm shift towards *improving healthspan* being as or even more important than increasing lifespan (Sierra, 2009; Murphy, 2011; Fries, 1980). In order to address the translation of therapies from the bench to bedside, critical elements must be in place such as animal models, experimental protocols, and assessment tools that evaluate interventions designed to improve healthspan (Kirkland, 2009). Moreover, to date, there is no assessment tool to evaluate neuromuscular

function in the mouse (healthspan), which would address the component of sarcopenia.

A common challenge in interpreting aging research is the wide statistical variability within outcome measurement data. The standard deviation (SD), commonly used to measure data variability, is a component of the standard error (SE), which plays a major role in determining statistically significant differences between experimental groups. The coefficient of variation (CV) is defined as the SD divided by the mean of the measurement. The CV thus describes the spread of the data in relation to the mean. However, mathematical constructs, such as multiple regressions, are used to reduce the effect of data spread by limiting the impact of covariates. Therefore, creating mathematical constructs to ameliorate statistical variation is one way to address wide data spread within groups in aging studies.

The primary purpose of this study was to develop a **N**euro**M**uscular **H**ealthspan **S**coring **S**ystem (NMHSS) that described healthspan in the C57BL/6 mouse and reduced the effect of variability within the selected outcome measures. Specifically, we hypothesized that the large individual variability of an outcome measure within groups that is often a bane to detecting treatment effects in aging studies would be attenuated by this scoring system. Therefore, the first step was to evaluate the performance and muscle contractility changes that occur with age

in the mouse model to select the determinants of the scoring system. Then, we constructed the scoring system based upon the age-group means and scores predicted by equations developed from multiple linear regression analysis of each determinant. Utilizing these multiple regression equations as a component within the scoring system allowed us to lower the CV of the NMHSS in comparison to the individual determinants.

Methods:

Animals:

Male C57BL/6 mice, from the NIA aging colony, of three specific age groups (adults, n=20, 100% survival, 6-7 months old; old, n=12, 75% survival, 24-26 months; and elderly, n=23, <50% survival, 28+ months) were selected for initial analysis (NIA, 2012; Miller, 2000). These age groups are translatable to human ages of young adult, middle age and elderly (NIA, 2012; Arias, 2007). Additional ages (from 2-32 months) were used to establish the multiple linear regression models for the NMHSS. In total, 70 animals were tested for contractile physiology and 99 for each of the performance tests (Rota-Rod and grip test). Animals were housed in a central specific pathogen free facility and experiments were conducted under IUCAC approved protocols ensuring optimal ethical and humane treatment. Because of animals lost to natural death and other variables, not all animals were tested in all parameters. Body and heart mass were determined on day of sacrifice.

Functional Measurements:

Rota-Rod:

Rota-Rod testing is a well-established measure of overall motor function (Ingram, 1986; Ingram, 1982). The mice are placed on a rotating cylinder (Pan Lab Lsi Rota-rod /RS Model 8200) and the time spent or duration, prior to falling is recorded. The device is set to either a run (static revolutions per minute-RPM) or

an accelerating (RPM consistently increases over time--accelerates from 4 to 40 RPM in 30 seconds, 2, 5 or 10 minutes) protocol. The mice were acclimated to the Rota-Rod prior to testing in order to familiarize the mice with the device and test protocols. Briefly, each mouse was acclimated on the Rota-Rod device for three trials per day on three consecutive days. On day 4, the performance of the mouse was tested using an accelerating protocol (5-minute slope to a max of 40 rpm) and the time the mouse remained on the Rota-Rod was recorded. This accelerating protocol was performed 3 times and the average of the 3 trials was calculated.

Inverted Cling Grip Test:

The inverted cling grip test is a measure of overall strength and muscular endurance of the mouse (Brooks, 2009). This test consisted of placing the mouse on a cage-like wire grid and then inverting the grid (over a padded surface).

A custom-designed device was constructed for the grip test to ensure consistency between trials. The mice were placed on the grid, which was located on the hinged lid of the device. The grid was held perpendicular to the device for 3 seconds, and then the lid was closed so that the mouse was inverted. The time the mouse held on before falling to the padded surface at the bottom of the device was recorded to a maximum of 180 seconds. The results were averaged between two trials.

Whole Muscle Physiology:

An *in vitro* isolated muscle preparation was used to measure the contractile properties of the extensor digitorum longus (EDL) muscles. Immediately after being weighed (massed), the EDL muscle was placed into a tissue bath filled with Krebs-Ringer buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose, pH 7.3), oxygenated (carboxygen: 5% CO^2 and 95% oxygen) and maintained at 25°C via a circulating water system. The muscle, viewed under a 1.75x swing arm magnifying glass, was tied with #4 suture line at both myotendinous junctions and then suspended from a force transducer and static clamp between two platinum electrodes in an oxygenated bath filled with Krebs buffer. The muscle was then tested for contractile function.

Equipment and Settings for Measuring Muscle Contractile Function:

A dual bath physiology system (Aurora Scientific, Aurora, Ontario, Canada) was used to measure isolated whole muscle contractility. This system consisted of two force transducers (0.5 N, Model 300B), two stimulators (Model 701B), one Dual Lever A/D Interface (Model 604B), one Dual System Signal Interface, customized Aurora software [Dynamic Muscle Control (DMC, version 4.1.4.6), Dynamic Muscle Analysis (DMA, version 3.2), and a temperature control with

water bath unit (Model 912 Polyscience Inc., Niles, IL**)**. The physiological system was controlled from a dedicated Windows compatible computer. The stimulators were set to bi-phase modality and the electrical output to 1000 milliAmps at 30 Volts.

Determination of Muscle Optimal Length:

Optimal muscle length (L_0) was determined using the peak twitch force-length curve. Briefly, the muscle length was increased until the peak twitch force (P_t) was achieved**.** Subsequently, the muscle length was measured from myotendinous junction to myotendinous junction using calipers. This measurement is L_0 (optimal length). L_0 is the muscle length where the sarcomeres are at optimal length to produce maximal force (Brooks, 1988).

Determination of Maximum Isometric Force:

The force-frequency curve was used to obtain the maximum or peak isometric force. Briefly, isometric force, with the muscle set at L_0, was determined at various frequencies (hertz, Hz): a single pulse (twitch), 10 Hz, 40 Hz, 80 Hz, 120 Hz, 150 Hz, 180 Hz and then again for a single twitch. The pulse length was 300 microseconds, software controlled by DMC. Each contraction was preceded by a one-minute rest period. The maximum isometric force (P_0) was defined as the peak force generated and was generally achieved at 150 Hz for the EDL.

Neuromuscular Healthspan Scoring System:

The NMHSS is an index comprised of a composite of three scores from the individual outcome determinants (Rota-Rod, grip test and EDL maximum isometric force). Prior to determining the composite of scores for the NMHSS, it was necessary to determine two important mathematical terms: (1) the statistical mean of each outcome determinant by age group, defined as the mean, and (2) the predicted outcome measurement for each determinant, which was calculated using a multiple linear regression equation. Next, the score for each outcome determinant was calculated from two components. The first component was the ratio of the actual measurement/mean for the age group and the second component was the ratio of the actual measurement/predicted measurement for the age group. Subsequently, the score for each determinant was defined as the mean of the two ratios. Finally, the overall healthspan score (NMHSS) was the sum of the three individual outcome scores (Rota-Rod, grip test and EDL maximum isometric force). **Figure 1** shows an example of how to create a Rota-Rod score using a representative mouse from the elderly age group.

The NMHSS identifies how different the animal's actual performance was from the group mean, identifies how far the animal's actual performance is from the predicted, and accounts for variability within each outcome measure. An animal

whose performance score is at the mean for all three determinants (actual measurement=mean and actual measurement=predicted measurement) will have a NMHSS of “3”. NMHSS > than 3 would suggest a healthier animal compared to an animal with a NMHSS < 3. **Table 1** illustrates the use of the Healthspan Scoring System with two examples of “old” mice (one with a low grip test and one with a high grip test) from our actual population and a fictitious average old mouse. The data for the fictitious average old mouse were derived from our reference group for each parameter.

Statistical Analysis:

Statistical differences between means were determined by One-way ANOVA and ANCOVA. Data are presented as means ± standard error (SEM) when appropriate. Significance was set at α=0.05 for ANOVA, ANCOVA, logistic regressions and simple/multiple linear regressions. The post-hoc test for ANOVA was Tukey-Kramer Honestly Significant Difference and a Bonferroni Correction was used for ANCOVA. Factor analysis using a promax rotation to determine principle components contributing to the variability of each set of outcome measures was performed. SPSS (IBM Corporation, Armonk, New York) used for statistical analysis aside from the power analyses (for a 3x2 ANOVA to detect a 15% treatment effect at 80% power), which were conducted with the PASS software package (from NCSS LLC, Kaysville, Utah).

Results:

Functional Measurements:

Rota-Rod:

The effect of age on overall motor function is summarized in **Figures 2, 3, and 4.** The mean Rota-Rod performance, the time spent on the rod during an accelerating protocol, declined 40% across the age groups (adult versus elderly p= 0.002; old versus elderly p=0.037, **Figure 2**). Moreover, there is a negative correlation between age and Rota-Rod performance (R= -0.373 p=0.001, **Figure 3**) explaining 14% of the variability (r^2=0.137). **Figure 3** shows poor Rota-Rod performance, represented as a 'dip', in mice between the ages of 12 and 20 months. Although the simple linear regression showed a negative correlation between age and Rota-Rod performance, the 3rd degree polynomial regression better explained the reduced performance at the middle age groups and the increased performance at the youngest and oldest ages.

In order to determine if Rota-Rod performance predicts the age of the mouse, a simple logistic regression (r^2=0.19, X^2=12.1, p=0.002) was calculated. The regression model was Adult (1) =reference group, Old (2): 1.734 - 0.014*Rota-Rod time, Elderly (3): 2.903 - 0.042*Rota-Rod time. The logistic regression correctly classified the mice into their proper age groups 57% of the time.

Figure 4 highlights the variability between individual mice and shows the broad range of ability to stay on the Rota-Rod within each of the age groups. Adult mice were able to remain on the accelerating Rota-Rod from 31 to 167 seconds, whereas the elderly mice remain from 1 to 116 seconds. To determine the major contributors to the variability, factor analysis and hierarchical multiple regression analysis were performed among all the experimental parameters. The multiple linear regression analysis showed that 52% of the Rota-Rod variability could be explained by body mass and heart mass ([n=35, R=0.720, r^2=0.518, p<0.001, model equation: Rota-Rod (s) = 237.951 - 1.798 body mass (g) -291.995 heart mass (mg)]). There was a positive correlation between age and body mass (R=0.429, p=0.010) in the animals that were Rota-Rod tested.

Using body mass as a covariant (ANCOVA, F=5.94 and p= 0.006) Rota-Rod performance was found to be statistically different between adult and old mice (Bonferroni-adjusted post-hoc test p=0.0332) and between adult and elderly mice (p= 0.011). There was no statistical difference between the old and elderly group.

Grip Test:

The effect of age on overall muscle strength, as measured by the inverted cling grip test, is summarized in **Figures 5, 6, and 7**. Using a one-way ANOVA to test the difference in mean performances, a significant age-related decline in grip strength was found between the adult and the elderly mice (61% reduction,

p=0.006) and between the adult and the old mice (39% reduction, p=0.002); however, there was no significant difference in performance between the old and elderly groups (**Figure 5**).

Using simple linear regression, grip test performance was found to be negatively correlated with age in months (R=-0.419) and 18% of the variability could be accounted for (r^2=0.176, p=0.001). The linear regression equation used was grip test (s) = -1.876 age (m) + 99.699 (**Figure 6**).

Figure 7 highlights the wide individual variability within each age group. In the adult group, the lowest functioning mouse held onto the grid for 41.5 seconds whereas the highest functioning mice held on for the maximum (180 seconds). In contrast to the adult group, the best performing mouse in the elderly group held on for only 94 seconds and the weakest mouse for 8.5 seconds. To determine whether the grip test performance can be used to classify the mice into their respective age groups even with this wide variation, without using any adjuster, a simple logistic regression was performed (**Figure 7**). The regression model used was: Adult (1) = reference group, Old (2) = 1.686 - 0.018*grip, Elderly (3) = 1.760 – 0.036*grip). The results indicate that the age group of the mouse is predicted correctly 56.6% of the time (r^2= 0.24, X^2=12.5, p=0.002).

To determine the major contributors to the variability in grip test, factor analysis and hierarchical multiple regression analysis were performed among all the experimental parameters. Age and body mass were the major sources of the variability. When corrected for age and body mass, 22% of the variability is accounted for [multiple linear regression, n=29, R=0.471; r^2=0.222; p=0.038, model equation: Grip Test(s) = 213.024 – 1.240 age @ grip test (m) -3.332 body mass (g)].

Muscle Contractile Physiology:

Utilizing *in vitro* methodology, we documented contractile properties for the EDL muscles, in the three age groups and along our healthspan continuum (2-32 months). The main contractile property investigated was peak tetanic force (P_0) of the EDL.

EDL Peak Tetanic Force (P_0):

EDL P_0 declined significantly with age (28%, p<0.001), from a mean of 388 mN in adult to a mean of 281 mN in the elderly (**Figure 8**). The decline from adult to old (mean 319 mN) trended towards significance (-18%, p=0.09), with no significant difference between the old and elderly (-12%, p=0.43). There was a correlation between age and EDL P_0 (R=-0.569). A simple linear regression of peak tetanic force of the EDL with the age in months (n=53, R=-0.569, r^2=0.324, p<0.001; with

the equation: P_0 = -4.452 age (m) + 420.128) showed that age accounted for 32% of the variability (**Figure 9**).

Figure 10 highlights the wide variability of EDL P_0 within each specific age group. For instance, the P_0 of the EDL from the adult group ranged from a high of 481 mN to a low of 286 mN, whereas the P_0 of the EDL muscles from the elderly group ranged from a high of 427 mN to a low of 182 mN. Notably, 35% of the mice from the elderly group performed better than the average mouse in the old group. Furthermore, 27% of the mice from the adult group performed worse than the average mouse in the old group.

To test whether the EDL P_0 was predictive of the mouse age group, a simple logistic regression was performed (r^2= 0.39, X^2=17.25, $p<0.001$). $P_{0\ classified}$ the animal into the elderly age group correctly 90% of the time using the following model equations: Adult (1) = reference group, Old (2): not significant, Elderly (3): $8.13 – 0.023*P_0$. In contrast, this regression model was not able to significantly classify the animal into the old age group. When using the old group as the reference, however, the regression model classifies the adult group 66% of the time.

In order to explain the individual variability within each age group a factor analysis was performed followed by a multiple linear regression using muscle

length and age as covariates. 46% of the variance (adjusted r squared = 0.46) was explained by the P_0 multiple regression equation [P_0 (mN) = 117.484 - 4.976 age (m) + 26.094 L_0 (mm)].

Relationship between the Outcome Measurements:

A simple linear regression of the determinants is shown in **Figure 11**, demonstrating that there was no significant correlation between Rota-Rod and EDL P_0 (or between force and grip test). There is a weak correlation, however, between Rota-Rod and grip test (r^2 =0.11). The three outcome measures are relatively independent measurements of neuromuscular health, each representing different or unique aspects of the mouse performance ability.

Neuromuscular Healthspan Scoring System:

The NMHSS for a cohort of mice is demonstrated in **Figure 12** (n=15 adult, n=5 old and n=19 elderly). The mean NMHSS score for the adult animals was 3.01 and ranged from 1.68 to 4.70 (standard error=0.196). The mean NMHSS score for the elderly animals was 3.05 and ranged from 1.71 to 4.44 (SE=0.154). In contrast, however, the mean NMHSS score for the old animals was 2.67 and ranged from 2.14 to 3.24 (SE= 0.211). **Figure 1** shows how the NMHSS is determined, using a single Rota-Rod raw score as an example.

In order to quantify and compare the amount of individual variability inherent within each age group, coefficients of variations (standard deviation/mean) were evaluated. When comparing the coefficient of variation of the NMHSS with the CV of our outcome measures, there was a reduction in variation of 2-fold for Rota-Rod and 3.7 fold for grip test; although there was a slight increase (0.23) from P_0.

To demonstrate the utility of the NMHSS ability to reduce variability, we compared power analyses (80% power to detect a 15% difference in a 3x2 ANOVA—designed as three age groups each with two treatment groups) using the mean of the elderly age group for each outcome measure (Rota-Rod, grip test, P_0). **Table 2** summarizes the results of the power analyses and the CVs. Notably, the number of animals needed for detection of the 15% difference is reduced by 77%, 87%, and 21% (for Rota-Rod, grip test, and P_0, respectively).

Discussion:

The purpose of this study was to develop a neuromuscular healthspan scoring system that will be used to evaluate treatments for sarcopenia. We hypothesized the wide variability within groups, which is often a bane to detecting treatment effects in aging studies, will be attenuated by this scoring system. The scoring system consisted of a mathematical construct designed as an index of determinants, which both compared the means of relevant outcome measurements and utilized multiple regressions to alleviate the effect of covariates. The main findings included a significant reduction in coefficient of variation with the NMHSS compared to the coefficients of variation of two of the outcome measures (Rota-Rod and grip test). This resulted in an increased ability to detect differences between groups, reflected by a reduction in the number of animals needed to detect a difference of 15% at 80% power in a 3x2 ANOVA in the NMHSS as compared to all three determinants alone. As expected, the main outcome measures (determinants: Rota-Rod, grip test, EDL P_0) declined with age (from adult to elderly--40%, -61% and -28%, respectively).

Scoring indices or testing batteries have been developed to measure frailty and to predict lifespan in the mouse (Ingram, 1986; Parks, 2012). In humans, there are multiple types of testing regimens designed to measure disability, frailty, mental health status and predict biological age (Whetstone, 2001; Pialoux, 2012; Cheung, 2012; Borkan, 1980). However, a neuromuscular healthspan scoring

system has not been developed. Neuromuscular health is defined as muscle force production combined with functional performance. This ability decreases, on average, in an age-dependent manner (Brooks, 1991; Narici, 2010; Wright, 2008). In the current study, our definition of neuromuscular healthspan is the ability to maintain an optimal level of performance (e.g., running, jumping) and strength/power output over the lifespan adequate to perform activities of daily living. Hence, the scoring system we developed quantifies neuromuscular healthspan within age groups.

The components of the NMHSS combine to present an overall picture of the neuromuscular health of the animal--both in comparison to the peers within its age group and in respect to what level of ability would be predicted by the multiple linear regression equation at the animals given age. Our mathematical construct (the mean of the ratios of the actual/mean and actual/predicted values) was a successful way to reduce variability of the individual determinants (Rota-Rod, grip test, P_0) by removing the effect of covariates using multiple linear regression (r^2= 0.52, 0.22, and 0.46 for Rota-Rod, grip test and P_0 respectively).

One significant outcome of reducing variability is lowering the effective SD by reducing the spread of data, which then reduces the se. This makes it easier to detect differences between group means because the 95% confidence interval around the mean becomes smaller—thus it becomes easier to achieve statistical

significance. Instead of reducing variability (SD), often the number of animals used (n) is increased to help achieve the same effect. In the current study, summarized in **Table 2** and with data taken from the actual cohort of elderly animals (**Figure 12**), the marked reduction in animals needed (sample size) is evident. Specifically, **Table 2** documents a power analysis by using NMHSS, for a 3x2 ANOVA to detect a 15% treatment effect at 80% power in the actual elderly group.

As noted in the Methods section, the NMHSS identifies how far the animal's actual performance is from the age group mean, identifies how far the animal's actual performance is from the predicted, and accounts for variability of each set of outcome measures. Assessing the relative neuromuscular health is another advantage of using the NMHSS. An animal whose performance score is at the mean for all three determinants (actual measurement=mean and actual measurement=predicted measurement) will have a NMHSS of "3". NMHSS > than 3 would suggest a healthier animal compared to an animal with a NMHSS < 3.

As highlighted in **Figure 12**, the mean NMHSS of the adult, old and elderly from the test cohort were 3.01, 2.67 and 3.05 respectively. The adult and elderly means indicated that the groups themselves tended to perform, on average, very close to what would be predicted. The old animals lower than 3 score can be

interpreted that the old group of animals performed, on average, at a lower than expected level.

Collectively, in the current study our young and elderly performed as expected whereas the old group was less capable. A unique application of the NMHSS is as assessment tool to describe the collective neuromuscular health of the age group cohorts and to describe the relative neuromuscular health of an individual mouse. Thus, there is a potential is to assess frailty within a group, by setting a cut-off value—below which an animal is considered frail or weak. For example, two standard deviations below the mean cohort NMHSS could be used to declare an animal a weak specimen within the group, whereas, two standard deviations below the average value of an age group (i.e. the old average animal NMHSS is 2.89, from **Table 1**) could be used to declare an animal frail.

Determinant Validity: Rota-Rod, Grip Test, EDL Peak Tetanic Force

One necessary component of scoring systems is the use of outcome measurements that ensure validity (Dodds, 1993; Sim, 1993, Ingram, 1983). Validity is defined as accurately measuring the intended measurement (Ingram, 1983). The determinants of the NMHSS were carefully selected and vetted to ensure maximum validity.

Rota-Rod and Neuromuscular Healthspan

We chose the Rota-Rod as our first determinant to measure overall motor function because it is one of the most common functional tests traditionally used for neuromuscular evaluation (Ingram, 1986; Ingram, 1982; Brooks, 2009). The outcome measurement is how long the animal can stay on the device. The mode of the Rota-Rod operation that we chose (acceleration) requires the animals to not only keep their balance and run on a spinning rod, but also to continually produce more power to keep up with the acceleration of the device. Because the animals will also tire from the exertion of the effort required to keep up with spinning rod, the measurement also contains a component of endurance. Therefore, this device demonstrates both face and content validity for our desired measurements (overall motor function: balance, coordination, gait speed, endurance) with only one measurement (time in seconds on device) because the time on the device directly correlates with the linear acceleration of the RPM. This outcome measure is similar to gait speed or timed-up-and-go testing used in humans, which are both used to document disability and frailty in humans (Whetstone, 2001).

We used the two variables, body and heart mass, that contributed most to the variability in the multiple linear regression. These two adjustors are appropriate and valid contributors to variability. Heart mass may very well be an indicator of underlying cardiovascular conditions. For example, an enlarged heart may signify

heart valve problems, cardiomyopathy, coronary artery disease or hypertension—any of which has potential to negatively affect cardiovascular performance (one aspect of the Rota-Rod test component that encompasses endurance) (Mackackova, 2006; Widmaier, 2006). Controlling for heart mass has the advantage of reducing the effect of these potential conditions on the outcome measurement. Bodyweight has obvious implications on performance because a large and/or obese mouse may not be as agile or as fatigue resistant in comparison to a mouse of normal weight.

Rota-Rod and the Survivor Effect

There was a clear dip in performance evidenced on **Figure 3**. The survivor effect may play a role in this dip. The survivor effect theory suggests that the strongest and healthiest animals will live to the oldest ages (less healthy/robust animals will succumb to disease prior to reaching the oldest ages); therefore, in some cases the performance of the older individuals will exceed that of their younger counterparts (Murphy, 2011).

Grip Test and Neuromuscular Healthspan

Another well-characterized measurement of neuromuscular ability is the grip test (Ingram, 1986; Ingram, 1982; Brooks, 2009). There are different ways to measure grip strength, including the use of force transducers attached to trapeze arms (the animal grasps the bar and is pulled backwards by the tail, outcome

being the force measured when the animal lets go) and suspending the mice from a grid and measuring how long they can hold on before they fall. While the former measurement has less involvement of muscle stamina and more directly measures strength, the latter gives information about both strength and endurance.

We therefore chose the inverted cling grip test to be our second functional test and built a custom testing device to ensure that the reliability of the test was maximized by making the conditions of each test identical. The face and content validity of the test are evident in that the outcome measurement (e.g., how long can the mouse suspend itself before falling) measures the ability of the mouse to support its body weight (strength) for a given amount of time (endurance). This outcome measure would be similar to a human pull-up test.

EDL Contractility and Neuromuscular Healthspan

One advantage of using the mouse model is that we can isolate individual muscles for whole muscle *in vitro* contractile physiology measurement. The EDL is primarily a fast fiber type muscle and thus is more sensitive to age-related muscle dysfunction (Brunner, 2007). P_0 represents raw force production—an absolute measurement of strength. This outcome measure is therefore somewhat comparable to a one-repetition maximum measurement of a weight

lifting exercise, but may be more reliable and valid because the muscle receives maximum stimulation since there is no voluntary component.

Conclusions:

Both functional ability and strength are impaired with age in the C57BL/6 mouse as evidenced by declines in grip test, Rota-Rod and EDL P_0. This was in agreement with other investigations (Ingram, 1986; Ingram, 1982; Brooks, 1988; Fahlstrom, 2012). There is, however, wide variation in the ability of individual animals. NMHSS, as a mathematical construct, is a much more sensitive instrument than the outcome measurements alone—due to lower coefficients of variation. This leads to an increase in power that allows detection of differences in means with ANOVA, while using far fewer animals than would be needed to detect the same difference in the separate outcome measures. The Neuromuscular Healthspan Scoring System may well become a very valuable tool for researchers to assess interventions in future studies.

In summary, the NMHSS reduces variability, increases power and serves as an assessment tool for neuromuscular ability. We postulate that in future investigations, the principles of the NMHSS may be adapted to producing other types of scoring systems in addition to providing researchers with a tool to assess sarcopenia interventions. By substituting other outcome measurements and by carefully considering validity, other types of scoring systems (e.g.,

cardiovascular, immune response, and others) could be produced using the principles behind the NMHSS.

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Legends

Figure 1: Production of Rota-Rod Raw Score. The figure shows an example of how to create a Rota-Rod raw score using a representative animal from the elderly age group (>28 months of age). Numbers in italics are the unique values associated with this animal. The first term of the equation is produced by taking the actual score of the animal in the test (*115* seconds, s) and then dividing by the mean score for the age group (60), which in this case equals 1.92. The second term of the equation consists of the actual score on the test (*115 s*) divided by the predicted score (*127.9 s*), which is produced by the model described by the multiple linear regression [n=35, R=0.720; r^2=0.518; p<0.001: model: Rota-Rod(s) = 237.951 - 1.798 Body Mass (*31.3g*) -291.995 Heart Mass (*0.184mg*) = *127.86*], which equals *0.899*. Both terms are then added *(1.92+0.899 = 2.819)* and divided by two to get the mean, *1.41*. This raw score for Rota-Rod, 1.41, is then added to the raw score for grip test and EDL force to obtain the overall NMHSS score. The same process is performed for each outcome measure. In the case of this particular mouse the grip test raw score was 0.709 and the EDL force raw score was 0.783. The total NMHSS score of this particular mouse was 2.902.

Figure 2: Rota-Rod performance declines with age. Rota-Rod performance, the time before falling, is defined as the average time in seconds (s) of three trials. The number of mice per group were 20, 32 and 13 for the adult (6 months, m), old (24 months, m) and elderly (>28 months, m), respectively. Values are means (100, 86, 60) for adult, old and elderly) ± SEM. Results are from a one-way ANOVA (F=6.3, p=0.003) with a Tukey-Kramer HSD post-hoc analysis. *: elderly significantly different from adult (p=0.002) and old (p=0.037).

Figure 3: Rota-Rod performance declines with age. This graph shows both a simple linear regression (r=-0.373, R^2=0.139, p=0.001, n=99; equation: y = -1.876x + 99.699), which explains 14% of the variability, and a 3rd degree polynomial regression (R=0.588, r^2 = 0.3453, p<0.001, n=99; equation: $y = -0.0157x^3 + 1.1005x^2 - 22.375x + 193.66$), in which 35% of the variation is explained. The 3rd degree polynomial regression demonstrates the survivor effect (animals at the oldest ages are healthier than some animals in middle age—i.e. 16-20 months). Note the dip in performance of the 16-20 month animals.

Figure 4: Large variability in Rota-Rod performance within each age group. Rota-Rod performance ranges from 31 to 167s in adult, 40 to 140s in old, and 1 to 116s in elderly mice. s: the average time in seconds (of three trials) spent on Rota-Rod before falling. 100, 86 and 60 represent the mean and the bar represents ± SEM. m: months old. *: elderly significantly different from adult (p=0.002) and old (p=0.037).

Figure 5: Performance on grip test declines with age. Grip test is defined as the average time (duration in seconds, s) before falling from the grid. The numbers of animals per group were: 18, 20 and 8 for the adult (6 months old, m), old (24 months old, m) and elderly (>28 months old, m), respectively. Values are means (112, 68, 47 for adult, old and elderly) ± SEM. Results from a One-way ANOVA ($F=6.8$, $p=0.003$) with a Tukey-Kramer HSD post-hoc analysis show: *: Adult different from Old ($p=0.002$) and from Elderly ($p=0.006$).

Figure 6: Grip Test declines with age. This graph shows a simple linear regression ($R=-0.419$, $r^2 = 0.176$, $p=0.001$, $n=99$; equation: Grip (s) = -1.876 age (m) + 99.699), in which age explains 18% of the variation. s: seconds. m: months.

Figure 7: Grip test performance shows wide individual variation within each age group. Grip test performance ranges from 41.5 seconds (s) to the maximum 180s (adult), 3s to the maximum 180s (old), and 8.5s to 94s in the elderly group. 112, 68 and 47 represent the mean and the bar represents ± SEM. m: months old. *: significantly different (from adult) mean ($p<0.05$).

Figure 8: The peak force produced by the EDL muscle declines with age. The numbers of animals per group were 15, 7 and 20 for the adult, old and elderly (respectively). Means (in milliNewtons, mN) were 388, 319, 281 for adult, old and elderly ± SEM. Results are from a one-way ANOVA ($F= 10.16$, $p<0.001$) with a Tukey-Kramer HSD post-hoc analysis: adult different from old (#: $p=0.09$) and from elderly (*: $p<0.001$). m: months.

Figure 9: Force Generation by the EDL Muscle Declines with Age. This graph shows a simple linear regression ($n=53$, $R=-0.569$, $r^2=0.324$, $p<0.001$) with the equation: P_0 = -4.452 age (m) + 420.128, in which age explains 32% of the variability of P_0. mN: milliNewtons m: months.

Figure 10: Force Generation of the EDL shows wide variability within age groups. The P_0 ranges from 286 to 482, 174 to 392, and from 182 to 427 in the adult, old and elderly groups respectively. 388, 319, 281 represent the mean and the bar represents ± SEM for adult, old and elderly, respectively. m: months, mN: milliNewtons. *: significantly different ($p<0.05$) from adult.

Figure 11: Little Correlation Between Outcome Measures of Rota-Rod, Grip Test, and EDL Force. mN: milliNewtons and s: seconds. **Panel A:** Grip test and Rota-Rod are weakly correlated. Rota-Rod/Grip Test regression ($n=66$): Rota-Rod (s)= 0.281 Grip (s) + 55.078 $R=0.334$, $r^2=0.111$, $p=0.007$. **Panel B:** Rota-Rod is not significantly correlated with EDL force. Rota-Rod /EDL force regression ($n=27$): P_0(mN) = -0.3107 Rota-Rod (s)

+ 382.84, R=0.232, r^2 =0.054, p=0.244. **Panel C:** Grip test is not significantly correlated with EDL force. Grip test/EDL force regression (n=30): P_0 (mN) = 0.1124 Grip (s) + 351.64 R=0.10, r^2=0.01, p=0.606.

Figure 12: NMHSS Scores for Adult, Old and Elderly Mice. NMHSS ranges from 1.68 to 4.7 (adult), 2.14 to 3.24 (old), and 1.7 to 4.44 in the elderly group. 3.01, 2.67 and 3.05 represent the mean and the bar represents ± SEM. m: months old.

Table 1: Comparison of NMMHS Score Construction. A: The table shows a comparison of the scores that comprise the NMHSS in two actual examples of mice from the old cohort (both 24 month old mice), one with a low performance in grip test and the other with a high performance. The first two columns describe the values that would be obtained by putting the mean values for the age group into the NMHSS equation. The derivation for each component of the NMHSS index follows the formula given in Figure 1. **B:** The values for the covariates used in multiple linear regression equations to produce the predicted terms are shown in this table. mm: millimeters. g: grams. * It should be noted that the fictional old mouse has values different from the mean of the old mouse cohort in Figure 12 because the values given are from the means for the entire sample of the population, not just the cohort described in Figure 12.

Table 2 NMHSS Reduces Effect of Variability. The coefficient of variation, CV, of the NMHSS is lower (0.22) than the CV of either the Rota-Rod (0.45) or the grip test (0.59). The end result is that the number of animals needed to achieve an 80% power (using means and SDs from the elderly cohort from Figure 12 in a 3x2 ANOVA at α=0.05 with the desired detectable difference being 15%) is much lower using the NMHSS (11) than the other tests (48, 82 and 14 for the Rota-Rod, grip and EDL P_0, respectively).

Table 1: Comparison of NMMHS Score Construction. A: The table shows a comparison of the scores that comprise the NMHSS in two actual examples of mice from the old cohort (both 24 month old mice), one with a low performance in grip test and the other with a high performance. The first two columns describe the values that would be obtained by putting the mean values for the age group into the NMHSS equation. The derivation for each component of the NMHSS index follows the formula given in Figure 1. **B:** The values for the covariates used in multiple linear regression equations to produce the predicted terms are shown in this table. mm: millimeters. g: grams. * It should be noted that the fictional old mouse has values different from the mean of the old mouse cohort in Figure 12 because the values given are from the means for the entire sample of the population, not just the cohort described in Figure 12.

Table 1

A.	Average Fictional Old Mouse (AF)*		Low Grip Old Mouse (LG)		High Grip Old Mouse (HG)	
	Mean	Score	Measure	Score	Measure	Score
Grip mean	68	1	34.5	0.51	116.5	1.71
Grip predicted	72.97	0.93	69.98	0.51	76.34	1.53
Grip raw score		**0.97**		**0.5**		**1.62**
P_0 mean	319	1	349.31	1.1	338.23	1.06
P_0 predicted	279.4	1.14	276.34	1.26	284.18	1.19
P_0 raw score		**1.07**		**1.18**		**1.13**
Rota-Rod (R) mean	86	1	64.7	0.7	57	0.61
R. predicted	122.2	0.70	117.78	0.55	132.42	0.43
R. raw score		**0.85**		**0.62**		**0.52**
NMHSS score		**2.89**		**2.3**		**3.27**

B.	Covariates	Mean	LG	HG
	L_0 (mm)	12.12	12	12.3
	Body mass (g)	33.1	34	32.09
	Heart mass (g)	0.19	0.2	0.16

Table 2 NMHSS Reduces Effect of Variability. The coefficient of variation, CV, of the NMHSS is lower (0.22) than the CV of either the Rota-Rod (0.45) or the grip test (0.59). The end result is that the number of animals needed to achieve an 80% power (using means and SDs from the elderly cohort from Figure 12 in a 3x2 ANOVA at α=0.05 with the desired detectable difference being 15%) is much lower using the NMHSS (11) than the other tests (48, 82 and 14 for the Rota-Rod, grip and EDL P_0, respectively).

Elderly Mice	SD	mean	CV	n (@ 80% power)
NMHSS	0.67	3.05	0.22	11
Rota-Rod	30.8	68.3	0.45	48
Grip	35.3	59.8	0.59	82
P_0	62.3	285.6	0.17	14

NMHSS = Sum of Raw Scores (Rota-Rod + Grip Test + EDL Force)

$$\text{Rota-Rod Raw Score} = \left(\frac{Actual}{\text{Mean}} + \frac{Actual}{\text{Predicted}}\right) \div 2 = \left(\frac{115}{60} + \frac{115}{127.9}\right) \div 2 = 1.41$$

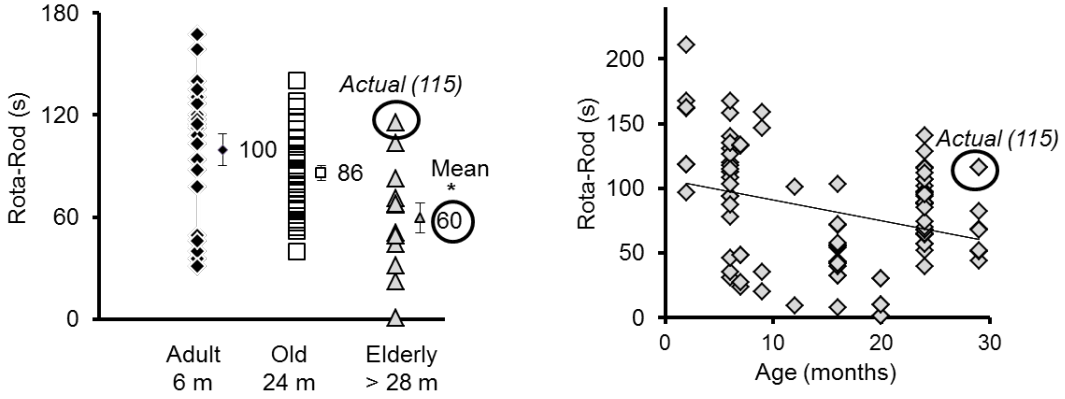

Figure 1: Production of Rota-Rod Raw Score. The figure shows an example of how to create a Rota-Rod raw score using a representative animal from the elderly age group (>28 months of age). Numbers in italics are the unique values associated with this animal. The first term of the equation is produced by taking the actual score of the animal in the test (*115* seconds, s) and then dividing by the mean score for the age group (60), which in this case equals 1.92. The second term of the equation consists of the actual score on the test (*115 s*) divided by the predicted score (*127.9 s*), which is produced by the model described by the multiple linear regression [n=35, R=0.720; r^2=0.518; p<0.001: model: Rota-Rod(s) = 237.951 - 1.798 Body Mass (*31.3g*) -291.995 Heart Mass (*0.184mg*) = *127.86*], which equals *0.899*. Both terms are then added *(1.92+0.899 = 2.819)* and divided by two to get the mean, *1.41*. This raw score for Rota-Rod, 1.41, is then added to the raw score for grip test and EDL force to obtain the overall NMHSS score. The same process is performed for each outcome measure. In the case of this particular mouse the grip test raw score was 0.709 and the EDL force raw score was 0.783. The total NMHSS score of this particular mouse was 2.902.

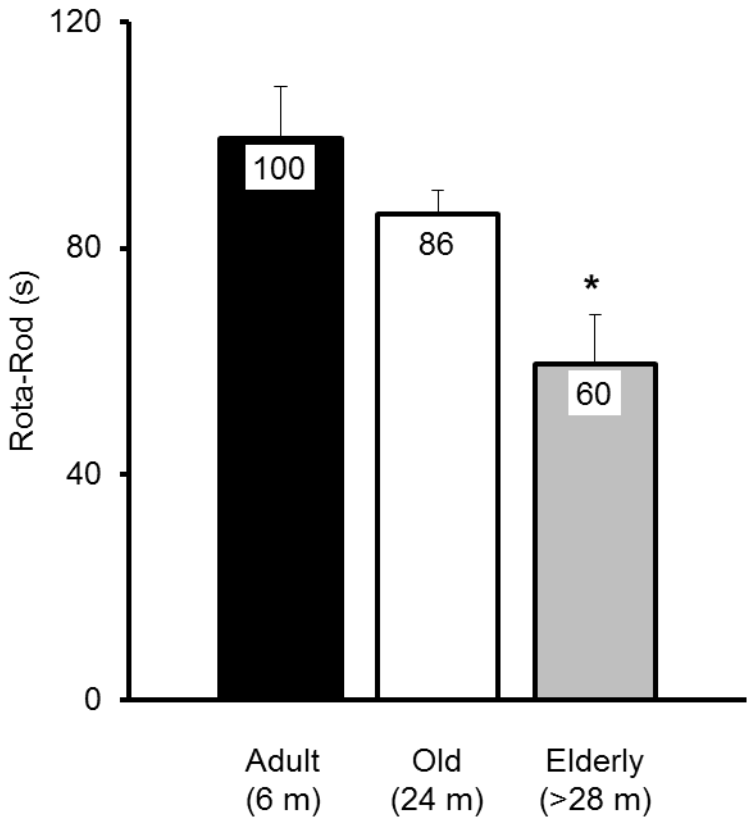

Figure 2: Rota-Rod performance declines with age. Rota-Rod performance, the time before falling, is defined as the average time in seconds (s) of three trials. The number of mice per group were 20, 32 and 13 for the adult (6 months, m), old (24 months, m) and elderly (>28 months, m), respectively. Values are means (100, 86, 60) for adult, old and elderly) ± SEM. Results are from a one-way ANOVA ($F=6.3$, $p=0.003$) with a Tukey-Kramer HSD post-hoc analysis. *: elderly significantly different from adult ($p=0.002$) and old ($p=0.037$).

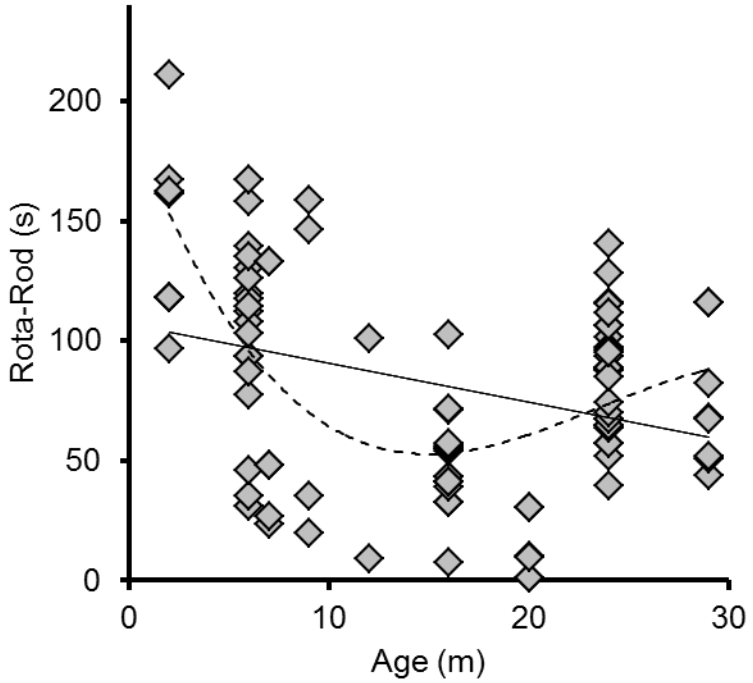

Figure 3: Rota-Rod performance declines with age. This graph shows both a simple linear regression (r=-0.373, R^2=0.139, p=0.001, n=99; equation: y = -1.876x + 99.699), which explains 14% of the variability, and a 3rd degree polynomial regression (R=0.588, r^2 = 0.3453, p<0.001, n=99; equation: $y = -0.0157x^3 + 1.1005x^2 - 22.375x + 193.66$), in which 35% of the variation is explained. The 3rd degree polynomial regression demonstrates the survivor effect (animals at the oldest ages are healthier than some animals in middle age—i.e. 16-20 months). Note the dip in performance of the 16-20 month animals.

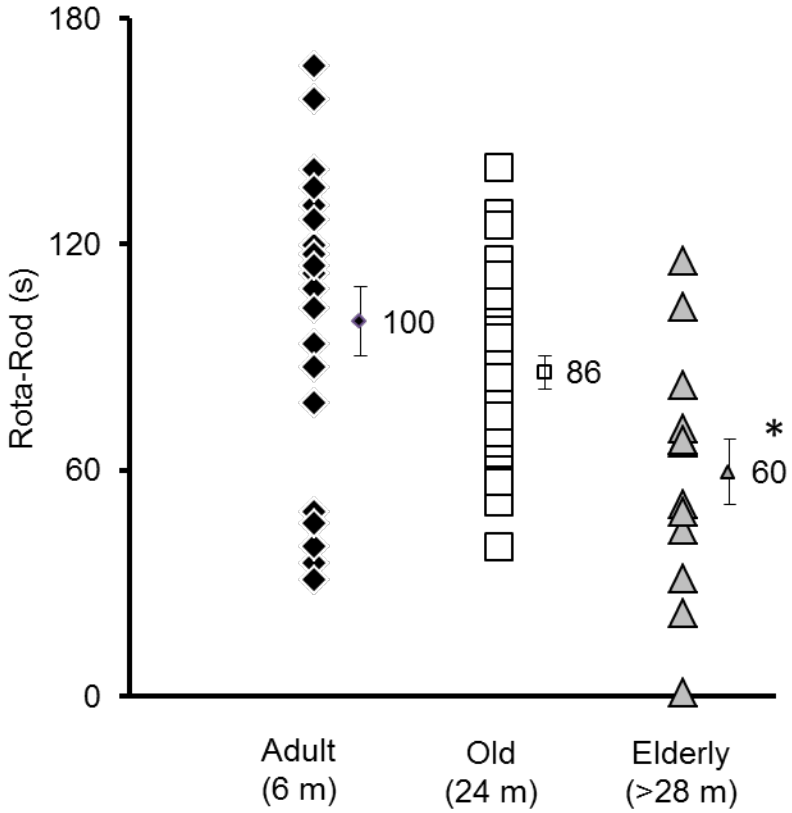

Figure 4: Large variability in Rota-Rod performance within each age group. Rota-Rod performance ranges from 31 to 167s in adult, 40 to 140s in old, and 1 to 116s in elderly mice. s: the average time in seconds (of three trials) spent on Rota-Rod before falling. 100, 86 and 60 represent the mean and the bar represents ± SEM. m: months old. *: elderly significantly different from adult (p=0.002) and old (p=0.037).

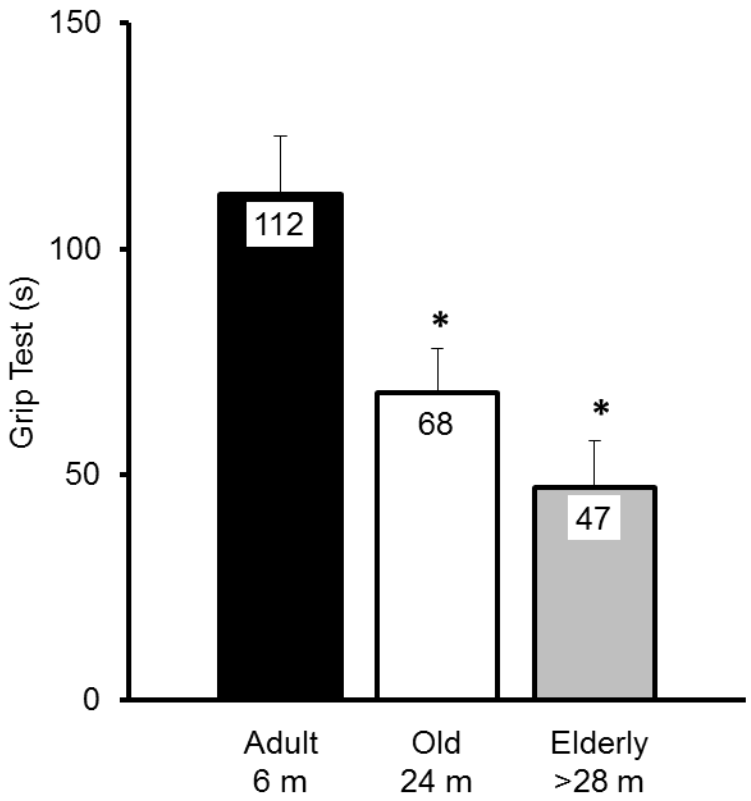

Figure 5: Performance on grip test declines with age. Grip test is defined as the average time (duration in seconds, s) before falling from the grid. The numbers of animals per group were: 18, 20 and 8 for the adult (6 months old, m), old (24 months old, m) and elderly (>28 months old, m), respectively. Values are means (112, 68, 47 for adult, old and elderly) ± SEM. Results from a One-way ANOVA ($F=6.8$, $p=0.003$) with a Tukey-Kramer HSD post-hoc analysis show: *: Adult different from Old ($p=0.002$) and from Elderly ($p=0.006$).

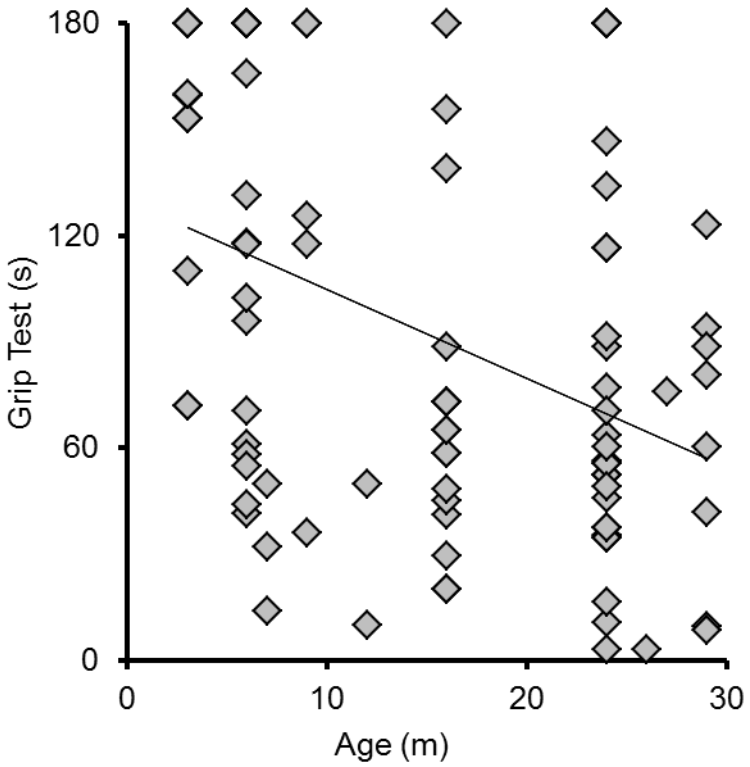

Figure 6: Grip Test declines with age. This graph shows a simple linear regression (R=-0.419, r^2 = 0.176, p=0.001, n=99; equation: Grip (s) = -1.876 age (m) + 99.699), in which age explains 18% of the variation. s: seconds. m: months.

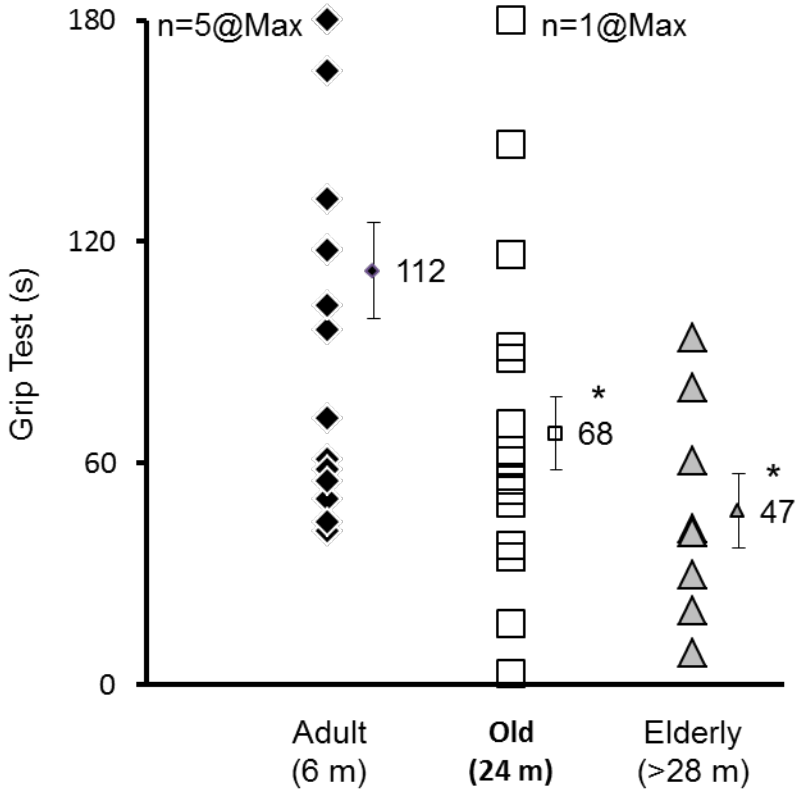

Figure 7: Grip test performance shows wide individual variation within each age group. Grip test performance ranges from 41.5 seconds (s) to the maximum 180s (adult), 3s to the maximum 180s (old), and 8.5s to 94s in the elderly group. 112, 68 and 47 represent the mean and the bar represents ± SEM. m: months old. *: significantly different (from adult) mean ($p<0.05$).

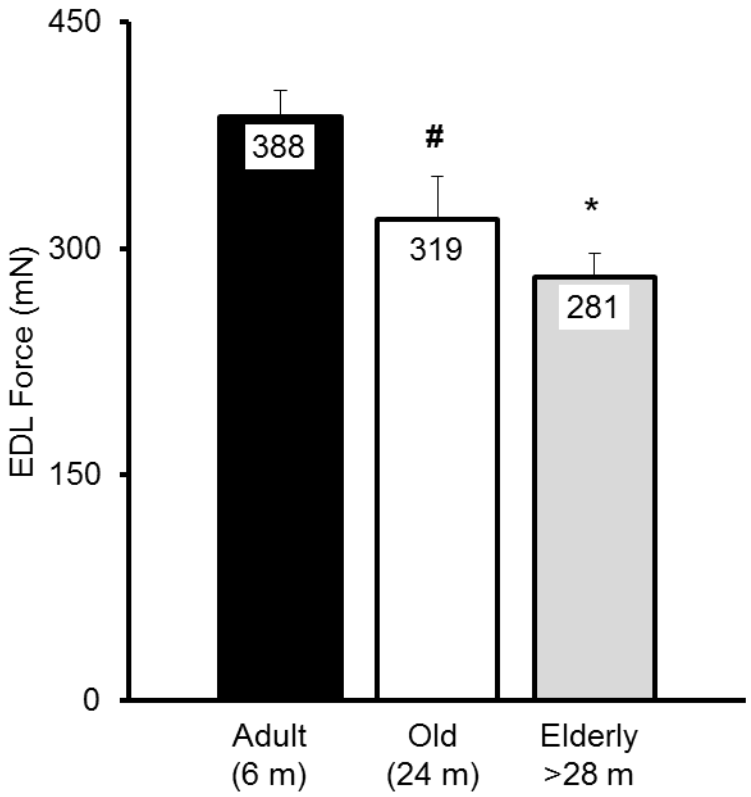

Figure 8: The peak force produced by the EDL muscle declines with age. The numbers of animals per group were 15, 7 and 20 for the adult, old and elderly (respectively). Means (in milliNewtons, mN) were 388, 319, 281 for adult, old and elderly ± SEM. Results are from a one-way ANOVA ($F= 10.16$, $p<0.001$) with a Tukey-Kramer HSD post-hoc analysis: adult different from old (#: $p=0.09$) and from elderly (*: $p<0.001$). m: months.

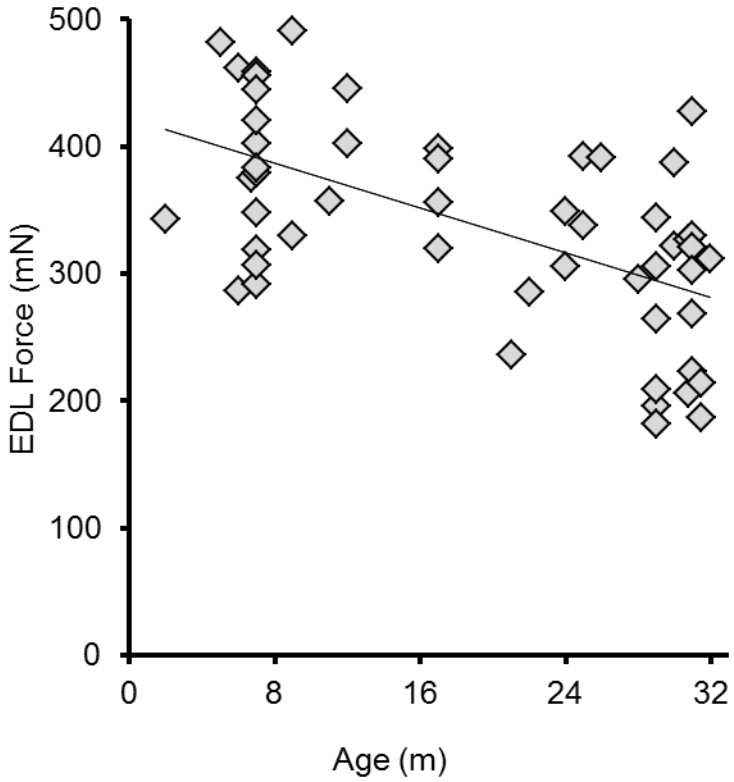

Figure 9: Force Generation by the EDL Muscle Declines with Age. This graph shows a simple linear regression (n=53, R=-0.569, r^2=0.324, p<0.001) with the equation: P_0 = -4.452 age (m) + 420.128, in which age explains 32% of the variability of P_0. mN: milliNewtons m: months.

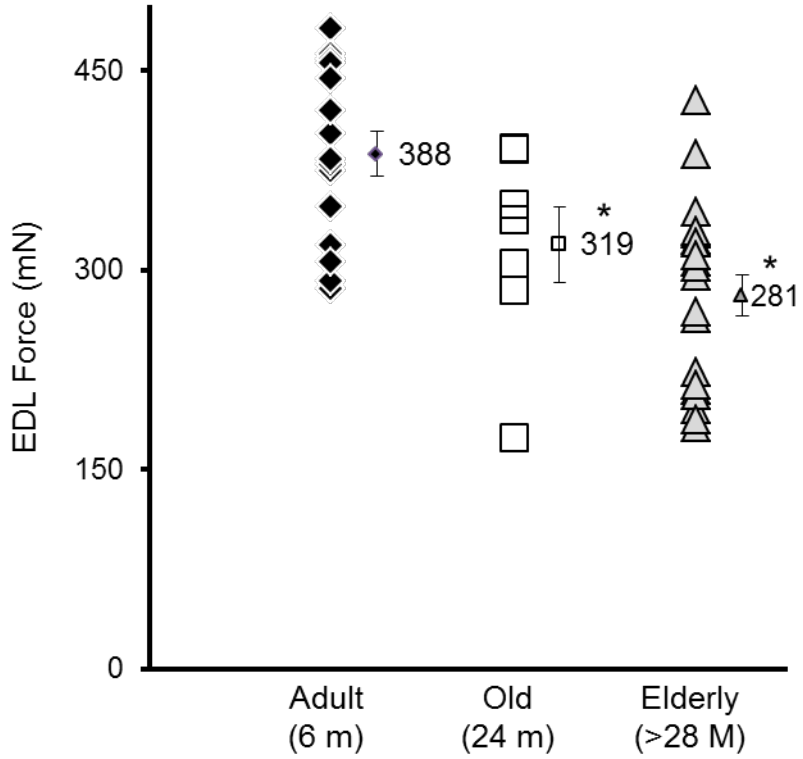

Figure 10: Force Generation of the EDL shows wide variability within age groups. The P_0 ranges from 286 to 482, 174 to 392, and from 182 to 427 in the adult, old and elderly groups respectively. 388, 319, 281 represent the mean and the bar represents ± SEM for adult, old and elderly, respectively. m: months, mN: milliNewtons. *: significantly different ($p<0.05$) from adult.

(s)

not

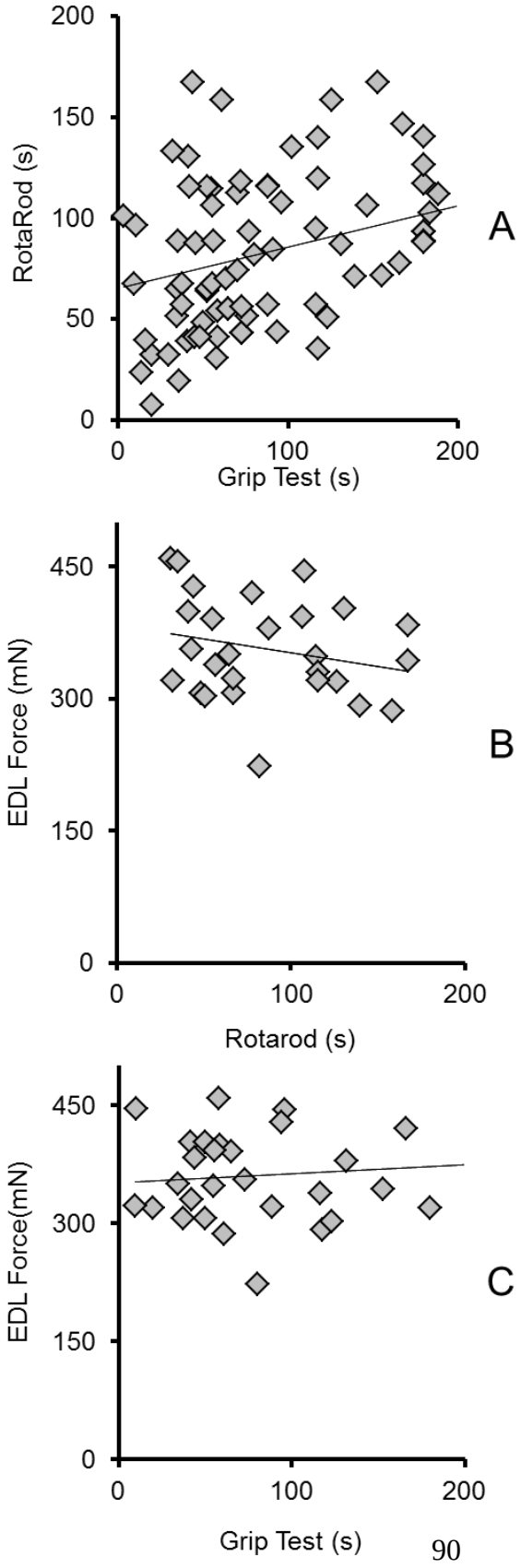

Figure 11: Little Correlation between Outcome Measures of Rota-Rod, Grip Test, and EDL Force. mN: milliNewtons and s: seconds. **Panel A:** Grip test and Rota-Rod are weakly correlated. Rota-Rod/Grip Test regression (n=66): Rota-Rod (s)= 0.281 Grip + 55.078 R=0.334, r^2=0.111, p=0.007. **Panel B:** Rota-Rod is significantly correlated with EDL force. Rota-Rod /EDL force regression (n=27): P_0(mN) = -0.3107 Rota-Rod (s) + 382.84, R=0.232, r^2 =0.054, p=0.244. **Panel C:** Grip test is not significantly correlated with EDL force. Grip test/EDL force regression (n=30): P_0 (mN) = 0.1124 Grip (s) + 351.64 R=0.10, r^2=0.01, p=0.606.

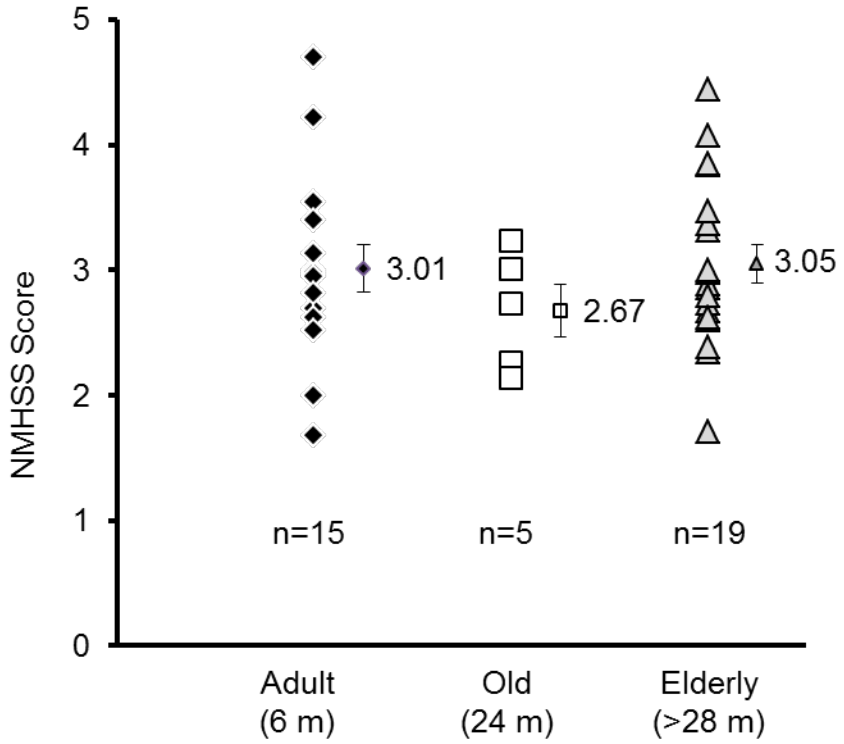

Figure 12: NMHSS Scores for Adult, Old and Elderly Mice. NMHSS ranges from 1.68 to 4.7 (adult), 2.14 to 3.24 (old), and 1.7 to 4.44 in the elderly group. 3.01, 2.67 and 3.05 represent the mean and the bar represents ± SEM. m: months old.

Interlude 1 Neuromuscular Healthspan and Contractile Dysfunction

In the previous chapter we learned that neuromotor function declined with age. There were age-associated deficits in maximum isometric force production (P_0), in the EDL, and lower times recorded on the rotarod and grip tests. However, there was individual variability that statistically obscured the ability to detect changes. Thus, the NeuroMuscular Healthspan Scoring System (NMHSS) was designed to reduce the effect of this variability. The NMHSS combined the three outcomes into a composite score. To produce the mathematical construct of the composite score each measurement was rated in relation to the mean predicted by a multiple regression analysis. The NMHSS greatly increased the power to detect change beyond that of the individual component outcome measurements alone.

Animals move with power (force multiplied by velocity), not force, thus the next part of this story was to investigate the effect of age on contractile velocity and power production. We hypothesized age-associated deficits in both power and velocity and we suspected the age-related dysfunction would become disproportionately larger in more difficult tasks (concentric contraction at percentages of P_0--above 50%). Most previous studies examined velocity of contraction at ≤50% of P_0 to find maximum unloaded velocity and used V_{opt} (velocity of contraction that produced P_{max}) to find P_{max}. Chapter 3 expands upon

the literature by probing into these unanswered questions (i.e. the effect of age on contraction under heavier loads) and extends our knowledge from Chapter 2 about the extent of contractile dysfunction with age, its possible etiology, and implications.

Chapter 2

This is a pre-copy-editing, author-produced PDF of an article submitted for publication following peer review.

C57BL/6 Lifespan Study: Age-Related Declines in Muscle Power Production and Contractile Velocity

Ted G. Graber[1], Jong-Hee Kim[1], Robert W. Grange[2], Linda K. McLoon[3], LaDora V. Thompson[1]

[1]University of Minnesota Medical School, Minneapolis, Minnesota 55455
Dept. of Physical Medicine and Rehabilitation

[2] Virginia Tech University, Blacksburg, Virginia 24061
Dept. of Human Nutrition, Foods, and Exercise

[3]University of Minnesota Medical School, Minneapolis, Minnesota 55455
Dept. of Ophthalmology and Visual Neurosciences

Corresponding Author:

LaDora V Thompson, thomp067@umn.edu, 612-626-5271 (office), 612-624-2436 (fax)
University of Minnesota Medical School
Department of Physical Medicine and Rehabilitation
Program in Physical Therapy
Rm 366A Children's Rehab Center
426 Church Street SE
Minneapolis, MN 55455

Running Title: Power and Velocity Decline With Age in Mice

Abstract:
Quantification of key outcome measures in animal models of aging is an important step preceding intervention testing. One such measurement, skeletal muscle power generation (force * velocity) is critical for dynamic movement. Prior research focused on maximum power (P_{max}), which occurs around 30-40% of maximum load. However, movement occurs over the entire load range. Thus, the primary purpose of this study was to determine the effect of age on power generation during concentric contractions in the EDL and soleus muscles over the load range from 10-90% of peak isometric tetanic force (P_0). Adult, old and elderly male C57BL/6 mice were examined for contractile function (6-7 months old, 100% survival; ~24 months and 75%; and ~28 months, <50%, respectively). Mice at other ages (5-32 months) were also tested for regression modeling. We hypothesized, and found, that power decreased with age, not only at P_{max}, but also, over the load range. Importantly, we found greater age-associated deficits in both power and velocity when the muscles were contracting concentrically against heavy loads (>50% P_0). The shape of the force-velocity curve also altered with age (a/P_0 increased). In addition, there were increased contraction times to maximum force and shifts in the distribution of the myosin light and heavy chain isoforms in the EDL. The results demonstrate that age-associated difficulty in movement during challenging tasks is likely due, in addition to overall reduced force output, to an accelerated deterioration of power production and contractile velocity under heavily loaded conditions.

Key Words: Mice, Sarcopenia, Muscle, Contractile Physiology, Power, Velocity

Introduction

One major health challenge facing our aging population is sarcopenia, the age-related loss of muscle mass and strength (Thompson, 2009). Sarcopenia not only decreases quality of life and makes activities of daily living more difficult, but it leads to loss of independence and is a major component of frailty (Landi, 2013; Fielding, 2011). Frailty is a syndrome characterized by low health reserve, decreased strength, generally poor prognosis for outcomes after medical procedures and increased mortality (Mackley, 2013). There is no cure for sarcopenia, though exercise (resistance training in particular) does show promise as a treatment because it increases muscle mass, strength, and function (Chodzko, 2009; Fiaterone, 1990; Evans, 1998). Movement requires skeletal muscle power generation. Skeletal muscle power is the product of two contractile parameters: force and velocity.

Research in mice to determine contractile properties *in vitro* has traditionally focused on P_0 (maximum isometric force production), V_{max} (a non-physiological measure of unloaded velocity derived from curve-fitting force-velocity data with the Hill equation), and P_{max} (maximum power, generally achieved at 30-40% P_0). P_0 and P_{max} decline with age (Lynch, 2001; Brooks, 1988 A, B), whereas an age-associated decrease in V_{max} has not been reported (Brooks, 1988 A). Similar to mice, P_{max} and maximum torque (strength) decline with age in humans, when measured as a joint movement (Reid, 2014; Narici, 1991). Plantar flexor motion demonstrates a decrease in both power and angular velocity with age in humans

(Clemencon, 2008). Therefore, it has been suggested that the loss of power production in the elderly is due to declines in both angular velocity and torque (Power, 2013). Human studies are necessarily performed *in vivo*, but in the mouse model it is possible to reduce the complexity of the *in vivo* system to a single muscle using the *in vitro* method.

P_{max} occurs within a narrow range of $\%P_0$, which does not encompass all types of dynamic movement. The force-power curve of a muscle is a hyperbola that peaks at 30-40 $\%P_0$ (P_{max}). Zero power is produced at zero load ($0\%P_0$), a hypothetical value because a muscle is always physiologically loaded. Power production is also 0 at 100% of P_0 (isometric contraction). Movement and physical performance require power production, and in the elderly a decline in power production represents an important indicator of physical function (Reid, 2012). Many functional activities require power production that spans the breadth of the force-power curve. Examples of human movement at the low range of the force-power curve include picking up a cup of coffee, or folding a shirt; whereas, at the high end activities such as opening a tightly sealed jar or arresting a fall require power production at forces closer to maximal P_0.

The emphasis on power production over the full force-power curve, by determining contractile velocity from 10-90% P_0, is an important expansion of the previously published *in vitro* contractility literature. For instance, Lynch et al. 2001 determined P_{max} using V_{opt} (maximum contractile velocity that produces the

maximum power output) and Brooks et al. 1988 determined contractile velocity at <50% P_0 in order to determine $V_{max.}$ Therefore, the primary purpose of the current study was to investigate power production in concentric muscle contractions against loads from light (10% P_0) to heavy (90% P_0) across the lifespan of the male C57BL/6 mouse, a commonly used aging model. The overall hypothesis was that power would decrease over the entire load range with age, with an age-associated loss of velocity, along with an overall reduction in force, both contributing to power reduction. Characterizing the effects of age on skeletal muscle power production over the entire load range and over the lifespan of the mouse will provide the foundation to design interventions for pre-clinical sarcopenia models.

To test our hypothesis, the maximum force (P_0) and the force-velocity relationship of both the soleus (SOL) and the extensor digitorum longus (EDL) were determined *in vitro*. The SOL is comprised of ~40% type 1 and 60% type 2 muscle fibers (Burkholder, 1994). The EDL is comprised of mainly type 2 muscle fibers (Burkholder, 1994). The force-power curve was then derived from the force-velocity relation. In addition, we tested for an age-related change in the distribution of myosin isoforms (myosin heavy chains and myosin light chains), since these proteins are major determinants of fiber contractile velocity (Moss, 1995; Larsson, 1997).

Methods

Animals:

Male C57BL/6 mice (n=80), from three age groups were analyzed [Adult (A, n=15; 5-7 months old, 100% survival), Old (O, n=14; 22-26 months, ~75% survival), and Elderly (E, n=24; 28+ months, <50% survival)] with additional mice outside of the 3 age groups used for regression analysis. All animals were aged from 5-32 months. The mice were obtained from the NIA aging colony at Charles River Laboratories (Charles River, Maryland). Some of these animals (n=50) were used in a previous study (Graber, 2013), where we reported peak tetanic force, rotarod, and grip test outcome measures. Mouse husbandry was performed by core staff in a specific pathogen free facility. IACUC approved standard operating procedures were used to assure humane and ethical treatment. Body and muscle mass were measured just prior to the physiology experiment, and body mass was also assessed 1 week after the mice arrived.

Whole Muscle Physiology:

Tissue handling, equipment and settings, determination of optimal length, peak twitch force, maximum tetanic force, and the force frequency curve are described in detail elsewhere (Graber, 2013). In brief, while under deep anesthesia, the SOL and EDL muscles were carefully removed from the animal and weighed. The muscles were tied with #4 silk suture just above their origin and insertion myotendinous junctions, and were attached vertically between platinum electrodes to a force transducer (300B, Aurora Scientific Inc. (ASI), Aurora

Ontario Canada) and a static clamp in a tissue chamber. The muscle was then submerged in Krebs-Ringer buffer in a vertical oxygenated bath (maintained at 25°C; Graber 2013). The muscle was stimulated (using 701B Stimulator, software controlled with Dynamic Muscle Control version 3.2; ASI) with a single pulse of 500 microseconds, at 30 volts and 1 amp, while length was increased until optimal length (L_0, measured with a caliper) was achieved--the point at which the peak twitch force (P_t) was determined. The muscles were then held at L_0 and stimulated at various frequencies, between 10 - 180 Hz, depending on muscle type to determine the peak tetanic force (P_0). Physiological cross-sectional area (PCSA) was determined using the average density of skeletal muscle by the formula: PCSA (cm^2) = Muscle mass (g) / [L_0 (cm) * 1.06 (g/cm^3)] (Mendez, 1960).

Force-Velocity Curve:

Using P_0 as the reference force, maintaining L_0 (optimal fiber length), and stimulating at the frequency where P_0 was found, the force velocity-curve was determined by using the load-clamp technique to measure velocity at various percentages of P_0 (10-90%). Specifically, the load-clamp technique sets the force transducer arm to resist at the $\%P_0$, but can travel freely once the muscle produces force that exceeds the load on the arm. The action can be thought of as moving against resistance, as in weight lifting. The muscle was maximally stimulated for 500 milliseconds (ms). The distance the arm moved over the time of stimulation was used to compute the maximum velocity at that $\%P_0$ by finding

where the first derivative of the time-distance curve equaled 0. The absolute measurement of velocity was in millimeters per second (mm/sec) which was converted to a normalized measurement of fiber lengths per second (fl/sec) using the established ratio values of intact fiber length to muscle length: 0.44 (EDL, adult), 0.45 (EDL, old and elderly), and 0.69 (SOL) (Brooks, 1988 A). The force-velocity curve was constructed, and the values entered into a custom MATLAB (Natick, MA) program. The custom MATLAB program fits the data to a curve using the Hill equation (Woledge, 1985), $(V+b)(P/P_0 \pm a/P_0)=b(1+a/P_0)$, to extrapolate the maximum unloaded velocity (V_{max}) and also plots a force-power curve (by fitting the data to a 5^{th} degree polynomial curve) to determine both the peak power (P_{max}) and the percentage of P_0 at P_{max} ($\%P_{max}$).

Force-Power Curve:

The power at the $\%P_0$ loads (force-power curve) was determined at the measured percentages of P_0 by multiplying force at $\%P_0$ and the maximum velocity at the $\%P_0$, determined as described above. The P_{max} and $\%P_{max}$ were taken from the MATLAB output, by determining the maximum point on the force-power curve.

Myosin Light Chain and Heavy Chain Isoforms:

This procedure has been previously described (Thompson, 2006). In brief, proteins from EDL and SOL samples, from whole muscle homogenates--made from muscles that were stored in skinning solution (which permeabalizes the

individual muscle fibers), were separated on large format electrophoresis gels at 12% acrylamide for light chain analysis and 5% for heavy chain analysis, and then silver stained. Proteins were positively identified by using tandem mass spec./mass spec. on a Thermo Scientific LTQ Orbitrap to identify the proteins on the standards. The standards were made from rat whole muscle homogenates (TA, SOL and diaphragm). The gels were imaged using a Bio-Rad Gel-Doc XRS Imager (Hercules, CA) and the relative protein abundance was calculated with densitometry using QuantityOne software (Biorad).

Statistics:

One-way ANOVA (Tukey-Kramer Honestly Significant Difference post-hoc) or Student's t-test were used to detect differences between means, as appropriate. Statistical significance was at $p<0.05$. Both simple and multiple linear regressions were analyzed to examine relationships between variables. General Linear model was used to compare slopes of regression lines. Principle component analysis using a promax rotation was used to determine the most significant variables for multiple regressions. We used SPSS (IBM Corporation, Armonk, New York) for all statistical analyses. Data are presented as means ± standard error (SEM).

Results:

Force-Velocity Relationship and Vmax:

The velocities of contraction under loaded (>0% P_0) conditions showed age-related declines (**Figure 1** and **Table 1)**. In the elderly mice, the extent of reduction in contractile velocities averaged 21±4% in the EDL (range of 10-34%) and 24±2% in the SOL (range of 16-33%); with no significant difference in the mean decline between the two muscles [Student's t-test comparing %reductions in velocity in elderly mice from 10-90%P_0: EDL and SOL; t=-1.1, p=0.290]. In the old mice, EDL velocities at 30-60%P_0 showed an age-associated reduction compared to the adult. For the old mice, there was slower contraction speed in the SOL compared to the adults from 10-80%P_0. Since the old mice were not significantly different from the elderly in contractile velocity, we report herein, for brevity, only the differences in velocity between adult and elderly mice.

Elderly EDL

The contractile velocities of the elderly, between 20% and 80% P_o, were significantly slower than the adult EDL group (**Figure 1 a, b and Table 1**). Specifically, at loads <50%P_0 (10, 20, 30, 40%) the velocities of the elderly group were reduced by 10 (not significant), 13, 15, 20% (p-values=0.051, 0.004, 0.001, 0.002), respectively. In contrast, at loads of 60, 80, and 90 (not significant) the velocities were reduced by 24, 34 and 34% (p-values=<0.001, 0.004, 0.064), respectively.

While V_{max} (unloaded velocity) did not change, age exerted a greater effect on EDL contractile velocity as the muscles contracted under increased loads (simple regression of the percent reduction and $\%P_0$: %Velocity= 0.33($\%P_0$) + 5.7, R=0.985) (data not shown). The mean velocity percentage (average percent change at all measured loads) was reduced 2-fold at heavier loads (40-90$\%P_0$) to 28%, in comparison to lighter loads (reduced 14% from 10-40$\%P_0$) (**Table 1 and Figure 2 a**). There was an additional age-related decline of 65% (slope of simple linear regression lines) at the heavier loads in comparison to the lighter loads [from separate linear regressions: %Velocity @ 10-40$\%P_0$= 0.23($\%P_0$) + 8.0, R=0.994; %Velocity @ 40-80$\%P_0$= 0.38($\%P_0$) + 2.1; R=0.972]. The slopes of these regression lines were different (p=0.003, from the General Linear Model) (**Figure 2**).

Elderly SOL

The SOL V_{max} was not significantly different in the elderly (-16%, ANOVA p=0.072, Tukey HSD p=0.061). However, the contraction velocity was significantly reduced by 23, 24, and 20%, at loads of 10, 20, and 30$\%P_0$, respectively. At the higher loads of 40, 60, 80 and 90$\%P_0$, the elderly mice contracted slower than the adults--27, 33, 33, and 31%, respectively (90$\%P_0$ not significant, p=0.081) (**Table 1 and Figure 1 c, d**).

Similar to the EDL, the effect of age became more pronounced at higher loads (simple linear regression of the percent reduction and 0-90$\%P_0$: %Velocity=

0.23(%P_0) + 16.5, R=0.946). The mean percent reduction in contraction velocity was 19% from 0-30%P_0, but was 30% from 40-90%P_0. When analyzing the regressions of the no/low load (0-40%P_0) and higher load (40-90%P_0) separately, the slopes of the lines were similar (suggesting a similar rate of effect), and R-values showed moderate correlation: %Velocity @ 0-40%P_0= 0.13(%P_0) + 17.3, R=0.757; %Velocity @ 40-90%P_0= 0.12(%P_0) + 16.5; R= 0.567.

Lifespan – Age and Velocities

In order to assess the effect of age, from 5-32 months, on velocity, we examined linear regressions of age with velocity at the various %P_0. EDL showed a significant negative correlation between age and velocity under loads from 20-90%P_0, whereas the SOL was significant at all loads from 10-90%P_0. All the linear regression graphs with equations are found in the **Online Resource 2 Supplemental Figures S1 and S2** [future references will use the convention "**Figure Sx**", representing the figure number in Online Resource 2 Supplemental Figures (**S1-S7**)].

Shape of Force-Velocity Curve, *a*/P_0:

To determine if there was a difference in the shape of the EDL and SOL force-velocity curves, the *a*/P_0 value was evaluated (*a*: constant from the Hill equation, *a*/P_0: describes the shape of the curve, see Methods). There was a downward and leftward shift with advancing age in the EDL (one-way ANOVA: F=19.1, p<0.001; Tukey HSD: adult vs. elderly p<0.001; old vs. elderly p=0.005) (**Table**

1). In contrast, the SOL curves did not exhibit a significant shift with age between groups (one-way ANOVA F=3.401, p=0.067) (**Table 1**). Over the lifespan, however, linear regressions showed an increase in a/P_0 with age in both the EDL and SOL, thus demonstrating that the force-velocity curve is altered with age (**Figure 3 a, b**).

Force-Power Relationship:

In order to determine the influence of age on power production, we constructed and analyzed force-power curves by multiplying the force produced and the velocity of contraction for both the SOL and EDL in the three age groups between 0-100%P_0, and then examined regressions of age (5-32 months) and power at all %P_0.

Power production declined with age (**Table 1**, **Figure 4, Figures S3 and S4)**. In the elderly mice, the reduction in power averaged 43±3% in the EDL (range of 33-54%) and 35±3% in the SOL (range of 31-39%), across the loads of 10-90%P_0 but there was no significant difference in percentage power reduction between the two muscles [Student's t-test: t=2.15, p=0.052]. In the old mice, the EDL had an overall power reduction of 20% (mean of all loads) and the SOL averaged 32% over the higher loads of 60-80%P_0. However, in the old SOL the decline observed from 10-40%P_0 was not significant, so we did not compare the old EDL to the old SOL with respect to the average percent change. Elderly mice had significantly reduced power at 10-80%P_0 (at 90%P_0, p=0.052) in the EDL,

averaging 29% and ranging from 25-41%, compared to old EDL. In the SOL, the old and elderly power production was not significantly different. In the interest of brevity, we will discuss in depth only the specific differences between the adult and elderly mice.

The percent reduction in power by the EDL per unit increase in load ($\%P_0$) had a linear relationship that amounted to -0.26% ($R=0.993$, $p<0.001$). Likewise, the SOL had a percentage decrease of -0.10 ($R=0.810$, $p=0.003$) in power, per unit increase in $\%P_0$. (**Figure 5 a and b**)

Elderly EDL

As noted above, power production was significantly reduced at loads between 10-90%P_o in the elderly EDL group. At loads <50%P_0 (10, 20, 30, 40) the power was reduced by 36, 38, 39, and 41%, respectively (**Table 1**, **Figures 2 B, 4 a, b and Figure 5 a**). In contrast, at loads of 60, 80, and 90%, power was reduced by 46, 53, and 54%, respectively. Age exerted a greater effect on EDL power production as the loads increased (simple regression of the percent reduction and $\%P_0$: %Power Reduction= $-0.26(\%P_0) - 31.3$, $R=0.991$, $p<0.001$) (**Figure 5 a**). Power was reduced 31% more at heavier loads (overall 51% mean reduction from 60-90%P_0) compared to lighter loads (overall 39% mean reduction from 10-40%P_0). The age-related decline was 57% more (comparing slopes of regression lines) in the heavier loads compared to the lighter loads [from separate linear regressions: %Power @ 10-40%P_0= $0.16(\%P_0) + 34.5$, $R=0.992$; %Power @ 40-

90%P = 0.28(%P_0) + 30.0; R= 0.992 (**Figure 2 b, Figure S4**). The slopes of the regression lines were different ($p<0.001$, from the General Linear Model).

Elderly SOL

Specifically, at the lighter loads of 10, 20, and 30%P_0, power was reduced by 31, 32, and 30%, respectively. At the higher loads of 40, 60, 80, and 90%P_0, power was reduced by 38, 35, 37, and 39%, respectively (**Table 1**, **Figures 4 c, d and 5 b**). As in the EDL, the effect of age became more pronounced at higher loads (simple linear regression of the percent reduction and 0-90%P_0: %Power= -0.10(%P_0) – 30.1, R=0.656, p=0.003) (Figure 5 B). The mean percent reduction in power was 31% from 10-30%P_0, but was 37% from 40-90%P_0. However, there was no indication of an increase in the rate of effect [from separate linear regressions of the no/low load (0-40P_0) and higher load (40-90%P_0), the slopes of the lines were similar, and R-values showed a moderately strong correlation: %Power @ 0-40%P_0 = 0.23(%P_0) + 25.5, R=0.769; %Power @ 40-90%P_0 = 0.21(%P_0) + 25.9; R= 0.819] (Data not shown).

Lifespan – Age and Power

To determine correlation and determine predictive equations, we examined linear regressions of age (from 5-32 months) with power at the various %P_0. Both the EDL and SOL showed significant negative correlations between age and power under all loads (**Figures S3 and S4**)

P_{max} and $\%P_0$ @ P_{max}

The EDL P_{max} of the elderly and old mice was reduced 40% ($p<0.001$) and 21% ($p=0.001$), respectively, when compared to the adults (**Table 1**). EDL P_{max} also declined 24% between old and elderly mice ($p=0.015$). Likewise, a simple linear regression of the EDL P_{max} with age revealed a significant decline [($R=-0.700$, $r^2=0.489$, $p<0.001$) with the equation: P_{max} (mN * fl/s) = 600.8 – 9.1 * age (m)] (**Figure S3**). The EDL $\%P_0$ @ P_{max} was reduced 13% ($p<0.001$) in the elderly (33%) and 8% (not significant, $p=0.069$) in the old (35%) compared to the adult (38%).

The SOL P_{max} reduced with age, 28% ($p=0.009$) in the elderly with no reduction in the old group (**Table 1**). Regression of the SOL P_{max} with age, however, revealed a significant decline over the lifespan ($R=-0.301$, $r^2=0.091$, $p=0.052$) with the equation: P_{max} (mN * FL/s) = 79.5 – 0.7 * age (m) (**Figure S4**). SOL $\%P_0$ @ P_{max} of the adult and elderly mice was not different (29%).

Relationships between Age, Force, Velocity, and Maximum Power

In order to evaluate the relationship between age, velocity, force and P_{max}, we examined simple and multiple linear regressions to determine how much variability is explained by the velocity and force components of power (equations and description of the procedures are found in the **Online Resource 1**

Appendix). In brief, in the EDL, P_0 explained 58% of the individual variability in P_{max} (P_0 + age, 66%), while velocity of contraction explained 23% (velocity + age, 49%). Both velocity and P_0 combined to account for 73% (with age, 75%). In the SOL, the P_0 could explain 59% of the individual variability in P_{max} (P_0 + age, not significantly different), velocity of contraction 50% (velocity + age, no difference), and P_0 and velocity combined, 83% (with age, 84%). (**Figure S5**)

Myosin Heavy and Light Chain Composition:

In the EDL, the percentage of myosin heavy chain (MHC) isoform 2a/x was increased, with the adults at 14% and the older mice at 20% of the total myosin, and the 2b isoform (adult 86% and older 80%) decreased by 6% with age, adults at 86% and old mice at 80% (bundle electrophoresis) (Student's t-test, p=0.009 and 0.010, respectively) (**Figure S6**). There was also a 21% decrease in the percentage of myosin light chain 3f in the EDL of older mice (combined old and elderly, n=23, mean age=28) when compared to adult (n=19, mean age=7.5) (**Figure S7,** Student's t-test, p=0.030). There was no difference in MHC 2a/x expression in the SOL (61.8% in adult and 61.0% in older mice, Student's t-test: p=0.670) or in MLC3f expression (11.0% in adult and 9.5% in older mice, Student's t-test: p=0.200) (**Figures S6 and S7**).

Animal Characteristics:

Table 2 summarizes the animal characteristics critical to interpreting the effect of age on muscle contractile velocity and power production. The body mass of the mice in the ~75% survival older group was heavier than both the 100% adult and

<50% elderly survival groups. Specifically, the body mass of the mice in the EDL old group was 17% heavier compared to the adults (p=0.008) and 13% greater than the elderly EDL group (p=0.018). In the SOL group, the old mice were 33% heavier when compared to the adult (p=0.001) and 27% more than the elderly (p=0.001). (ANOVA, with Tukey HSD posthoc)

EDL muscle mass and size decreased with age. EDL muscles in the elderly group had a 25% reduction in muscle mass (p<0.001) and a 27% reduction of the physiological cross-sectional area (PCSA, p<0.001) when compared to the EDL muscles in the adult mice. EDL muscle mass in the old group was not significantly different (-13%, p=0.085), but there was a 17% reduction in the PCSA, (p=0.039) compared to the adult mice. (ANOVA, with Tukey HSD posthoc)

When muscle mass was normalized to body mass (mg of muscle mass/grams of body mass, or mg/gbm), the reduction was confirmed. The old EDL (0.37±0.01 mg/gbm) were 25% smaller, and the elderly (0.35±0.01mg/gbm) were 28% smaller (ANOVA, p<0.001; Tukey HSD p=<0.001 for both).

In contrast to the EDL, the SOL muscle mass and PCSA did not show the age-related decrease. However, the normalized muscle mass revealed an age-related decline such that the SOL muscles from the old mice (0.31±0.03 mg/gbm) were 30% lighter, although muscles from the elderly mice (0.36±0.01mg/gbm)

were not significantly different (-29%) when compared to the adult group. (ANOVA, p=0.012; Tukey HSD p=0.009 and 0.077, respectively).

Force Production Declined with Age:

With age, the P_0 of the EDL was reduced **(Table 2)**. The elderly produced 28% less force ($p<0.001$) and old mice 22% less ($p=0.003$), in comparison to the adult. EDL P_0 normalized to muscle cross sectional area was not different. The normalized EDL P_0 (to body mass) was lower in both the elderly and old mice compared to adult by 29% ($p<0.001$) and 20% ($p<0.001$), respectively. In contrast, the SOL did not show a significant decline in P_0 ($p=0.057$). SOL also did not decline in P_0 normalized to muscle cross sectional area. However, after normalizing SOL P_0 to body mass, there was an age-related decline. The old mice (4.8±0.43 mN/gbm) lost 22% of normalized force in comparison to the adults (6.1±0.40 mN/gbm), but the elderly (5.1±0.23 mN/gbm) were not significantly different from the adults (ANOVA, p=0.042; Tukey HSD: Old p=0.043 and Elderly p=0.103).

To determine the effects of age on EDL P_0 over the lifespan (5-32 months of age), a simple linear regression of the P_0 with age revealed that age explained 30% of the variation in P_0 [$R=0.550$, $r^2=0.302$, $p<0.001$; equation: P_0 (mN) = 425.99 (mN) – 4.45 * age (m)]. This relationship of EDL and age was similar to the results we found previously (Graber, 2013). In contrast, neither the simple

regression with age of the SOL P_0 nor the normalized SOL P_0 were different (p=0.121 and 0.140, respectively).

In the EDL, the time to reach maximum force (contraction time, measured at 150 Hz, average frequency for P_0) was 0.159±0.008 sec, 0.178±0.007 sec, and 0.211±0.010 sec in adult, old and elderly, respectively (**Figure 6** shows regression in respect to age). There was no difference in the contraction time between the adult and old muscles, but the elderly had a contraction time 16% longer than the old (p=0.036) and 33% longer than the adult (p<0.001) (One-way ANOVA, Tukey HSD post-hoc). Across the lifespan (5-32 months, n=63), age had a modest negative correlation with EDL contraction time (R=0.525, p<0.001). In the SOL, there was no significant difference in contraction time (measured at 100 Hz).

Discussion:

The comprehensive analyses of power output and contractile velocity across the lifespan in both the EDL and SOL muscles provide novel insight into age-related contractile dysfunction. Overall, we found age-associated declines in power, velocity, and force in both the EDL and the SOL. The extent of decline varied with respect to $\%P_0$, as the velocity and power of both muscles were affected more by age when the muscles were contracting against the heavier loads (i.e., loads $>50\%P_0$). The age-associated loss of both power and velocity at heavy loads has important functional implications, particularly in activities in which a large proportion of body mass must be moved. Getting up from a chair, acceleration of gait, and ascending or descending stairs are examples. Time to maximum force in the EDL increased with age, as did a/P_0, a measure of the shape of the force-velocity curve. In addition, we found that decreases in velocity became a predictor of power loss as the load increased from light to heavy. Finally, in the EDL, with advancing age, there was a decline in the percentage of myosin light chain 3f (old<adult), and a decline in the relative MHC 2b content.

There were *three* major motivating factors for this investigation. *First*, although maximal power generation occurs at 30-40% P_0, functional activities require movement that incorporates power generation across a wide load range, especially power generation under heavily loaded conditions. Fall prevention is one critical heavily loaded condition where the rapid application of power to a limb is needed for an individual to regain balance. Other typical activities of daily

living that require power generation at large percentages of maximum force are the opening of a stuck jar lid or rising to a standing position from a seat. Such activities of daily life are compromised in the older adult (Suzuki, 2001; Raj, 2010). Indeed, the loss of power has been suggested to be a strong predictor of disability (Puthoff, 2007; Suzuki, 2001). Not only does the loss of power contribute to disability, but, importantly, the velocity of the contraction itself correlates with physical performance, and emphasizes the value of investigating this parameter under loaded conditions (Clemencon, 2008). *Second*, it is now well established in humans that resistance training with loads around 80% of peak strength maximizes strength gains and is considered the best-practice intervention for sarcopenia (Fiaterone, 1990; Evans, 1998; Evans, 1993; Ali, 2014). *Third*, studying basic contractile properties over the entire load range and how they change over the lifespan is necessary because the information obtained provides a better perspective of the activities of daily living that are likely impacted, especially those requiring muscle force production >50%P_o (i.e., heavy loads). In turn, this knowledge may lead to mechanistic investigations that provide targets pertinent to treatment strategies for sarcopenia that reverse frailty, increase power production, and improve quality of life in the elderly.

Interventions to prevent, reverse, or slow sarcopenia have mainly focused on strength gain and muscle hypertrophy. Based on the findings of this study, and assuming the results are translatable to humans, designing training programs for muscle power and velocity gains at higher percentages of maximum force would

meaningfully impact performance for the older adult. Previous reports show greater improvements in power production when training for velocity of contraction, power training, compared to training for strength (Earles, 2001). More importantly, functional ability is improved greatly with power training. For example, power training in community-dwelling older adults was shown to have a greater impact than strength training on functional ability, such as measured by the Continuous Scale Physical Functional Performance test (Miszko, 2003). Thus, some combination of exercise training strategies that include strength/hypertrophy, as well as power and velocity training, may be required to maximize functional improvement, and to mitigate sarcopenia/frailty. This combined exercise training strategy will likely require additional support through ergogenic and nutritional supplementation to assist in overcoming the anabolic resistance reported in the elderly and to maximize gains, since anabolic resistance has a multi-factorial etiology (Haran, 2012).

Peak values - P_{max}, V_{max}, and P_0:

Previous studies report the traditionally measured peak values of power (P_{max}), velocity (V_{max}), and force (P_0). With age, both P_{max} and P_0 decline (Lynch, 2001; Narici, 2005; Brooks, 1988 A and B; Graber, 2013; Phillips, 1991), whereas V_{max} (derived from the force-velocity curve) is relatively unaffected (Brooks, 1988 A; Lynch, 2001). Peak values in the current study are consistent with these results.

Our study found key differences in two contractility outcome measures, a/P_0 and contractile velocities over the load range (at both low and high loads). Our comprehensive analysis revealed that, with age, there were significant increases in the a/P_0 ratio, suggesting a change in the force-velocity relationship (Jones, 2010), as well as decreases in power production and contractile velocity. These differences likely are attributed to the age of the animals and to differences in the experimental design for evaluation of the force-velocity and force-power curves. Our oldest group of mice was 2-3 months older (mean age 30 months) than the oldest mice in previous studies (mean ages 27-28 months). In addition, previous studies only measured velocity at percentages of P_0 less than 50%.

The current study reports other novel findings: the impact of age on both velocity and power during heavily loaded contractions; the increased relationship (R value) between velocity and power at larger percentages of P_0; the reduced percentage of maximum force at which maximum power was produced in the EDL as a function of age; the increased contraction time to maximum force in the EDL; and the comprehensive characterization of the decline in velocity and power over the lifespan (5-32 months) of C57BL/6 mice via linear regression.

Mechanisms underlying age-related power impairment:

Any reduction in force or velocity will result in loss of power. The loss of force production is generally accepted to be associated with muscle atrophy, motor unit loss, and deficits in muscle quality (Raj, 2010). Changes in connective

tissue, such as a decrease in elastic modulus in tendons, (Onambele, 2006) and alterations in muscle architecture, such as pennation angle, are also likely to influence contractility negatively (Thom, 2007) by diminishing force transmission. However, the reduction in force capacity does not entirely explain the accelerated loss of power seen with age (Thom, 2005).

Our findings that the a/P_0 ratio increased with age also suggest a change in the force-velocity relationship (Jones, 2010). Age-related reduction in contraction velocity may also contribute to loss of power. In the human and rodent literature spanning various experimental technologies, such as single skeletal muscle fibers and applied human performance, an age-related loss of velocity has been reported (Thompson, 1999; Kim, 2012; Li, 1996; Krivikas, 2001; Larsson,1997; Narici, 1991; Thom, 2007), with some disagreement in studies of human single muscle fibers (Trappe, 2003). In numerous *in vivo* human studies, many potential contributors to loss of velocity and power have been suggested: neurological changes, motor unit recruitment (Narici, 2008), increased proportion of fat/connective tissue in older muscle (Addison, 2014), muscle architecture changes, including pennation angle and fiber fascicle length reductions (Klein, 2001), and deleterious joint/range of motion/mobility changes (Lanza, 2003).

Likely candidates for the age-related velocity decline within individual skeletal muscle fibers include impaired actin/myosin interactions (Hook, 2001; Raj, 2010) and changes in myosin heavy and light chain isoform proportions (Moss, 1995;

Larsson, 1997). The main factor that determines the velocity of a muscle cell contraction is the MHC isoform proportion/distribution of the fiber (Schiaffino, 1990). We found a small but significant age-associated shift in MHC content in the EDL with a 6% decline in MHC2b in the elderly mice that might explain some of the velocity change. Contractile velocity of a single fiber is also regulated both by the phosphorylation state of the regulatory MLC2 and the isoform composition of the essential light chains (Grange, 1995), with MLC3f being the fastest (Kim, 2012). We found a 21% decline in MLC3f relative content in EDL fibers that, together with the decrease in MHC2b, might partially explain the reduced velocity. In previous work from our laboratory, single fibers from rat semimembranosus, showed a 69% decline in %MLC3f content in MHC type II fibers with age. However, up-regulation of MLC3f using gene therapy restored single-fiber unloaded contractile velocity in older rats to adult levels (Kim, 2012). Further investigation is needed to clarify the roles of myosin isoform changes and their contributions to velocity and power loss.

Possible Mechanisms of Increased Age-Associated Dysfunction at Higher Loads:

At low loads, the muscle function was better preserved; however, at high loads, the impairments in myosin-actin interactions were exacerbated leading to reduced contractility. Indeed, myosin working stroke distance and velocity are reduced at higher loads (Reconditi, 2004). We believe that age-associated changes in actin-myosin cross-bridge kinetics, such as the ratio of strong and

weak structural states, resulted in fewer strong-binding attachments, decreased detachment rates, and increased internal drag, play a role in load-associated decline of function with age (Prochniewicz, 2005). Further investigation is needed.

Our finding of an age-related increase in EDL contraction time to maximum force suggests connective tissue dysfunction as an underlying cause of power impairment (Narici, 2008). At the tissue level, the tendons, myotendinous junction, and other connective tissues responsible for force transmission have reduced functionality with age (Onambele, 2006; Zhang, 2014). These age-related changes result in thickening of the extracellular matrix and detrimental mechanical alterations to the tendon, caused by a decrease in elastic modulus and stiffness resulting in increased compliance, which negatively alter force transmission speed and ultimately contractile velocity (McCarthy, 2014; LaCroix, 2013, Narici, 2008). We propose that these detrimental modifications in connective tissue are manifested at a greater extent and contribute to increased dysfunction when the muscle is contracting against higher load. Further investigation is needed to delineate the contribution of connective tissue decline to loss of contractile efficiency.

It is important to note that lifestyle and behavior may play a role in dysfunctional power production at heavy loads. As organisms age, not only does the general rate of activity decline, but the tendency to engage in behaviors that require near

maximal exertion declines as well (Jefferis, 2014). For example, muscles used for postural activities often have less age-related decline than muscles designed for explosive movements (SOL vs. EDL in the current study, for example). In addition, motor unit reorganization with age does not favor the maintenance of fast-twitch anaerobic activities, due to preferential type 2 motor unit net loss and the more general preservation of type 1 myosin muscle fibers with age (Luff, 1988; Thompson, 2006).

Conclusion:

There was an age-associated decline in power, velocity, and force in both the EDL and SOL muscles of the C57Bl/6 male mouse. Specifically, contractions at heavy compared to light loads showed an exacerbated age-associated decline of power and velocity. The shape of the force-velocity curve was also altered with age, with a/P_0 increasing. We also detected a reduction in EDL MLC3f and MHC2b content, which may contribute to the declining velocity of contraction. Our finding showing an age-related increase in time to maximum force in the EDL suggests that, among other potential mechanisms such as impaired calcium handling, connective tissue dysfunction may play a role in power and velocity loss. Further investigation is needed to determine the specific mechanisms underlying age-associated increased dysfunction in muscles contracting concentrically under heavy loaded conditions.

Author Contributions:

Ted G. Graber: primary study designer; performed all experimental data collection, data analysis/interpretation; primary writer of manuscript; email: grab0170@umn.edu

Jong-Hee Kim: provided assistance in designing Matlab software; assisted in data interpretation of the myosin light chain; scientific review of manuscript; email: jkim77@central.uh.edu

Robert W. Grange: provided expertise in the training of *in vitro* physiology experimentation; scientific review of manuscript; email: rgrange@vt.edu

Linda K. McLoon: provided expertise in data storage software; scientific review of manuscript, funding (hardware maintenance); email: mcloo001@umn.edu

LaDora V Thompson: responsible for all aspects of study, guarantor of the study, provided critical assistance in data interpretation and writing of manuscript, funding; email: thomp067@umn.edu.

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Jong-Hee Kim is currently at University of Houston, Houston Texas 77204, Dept. of Health and Human Performance.

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Table Legends:

Table 1 Power and Velocity at Percentages of Maximum Force (%P_0) V_{max}= maximum unloaded velocity. P_{max} = maximum power. %P_0@P_{max} = the percentage of maximum force where P_{max} was produced. mN * fl/s = milliNewtons multiplied by fiber lengths per second. P-value was taken from one-way ANOVA. %Change = % change from adult to elderly. Symbols: a=different than adult, b=different than old ($p<.05$) (marked only on old and elderly). § = P_{max} values from MatLab data curve-fit, force-power curve derived from the maximum instantaneous velocity (at %P_0) multiplied by force (at the %P_0). In the adult SOL, the derived P_{max} (81±6) is not statistically different than the derived value for power at 30%P_0 (82±6).

Table 2 Animal Characteristics for EDL (n=53) and SOL (n=47) Groups Adult mice (EDL n=15, SOL n=12) were used as the reference group to the old (EDL n=14, SOL n=12) and elderly (EDL n=24, SOL=23). P-value was taken from one-way ANOVA (Tukey's HSD post hoc test). PCSA= physiological cross-sectional area of muscle. P_0= maximum tetanic contractile force. Symbols: a=different than adult, b=different than old ($p<0.05$) marked only on old and elderly.

Figure Legends:

Figure 1 Velocity Declines with Age a) EDL Force-Velocity Curve b) Linear Regression of EDL velocity at 60%P_0 See **Figure S1** in the supplement for the other velocities. **c) SOL Force-Velocity Curve d) Linear Regression of SOL velocity at 60%P_0.** See **Figure S2** in the supplement for the other velocities. See **Table 2** and Text for post-hoc analysis. Each symbol in **b** and **d** (diamonds) in the regression graphs represents a measurement from an individual mouse at the given age. Equation: simple linear regression of velocity (y) as a function of age (x). ***** = $p<0.05$; p-value from One-Way ANOVA. fl/s= fiber length per second. %P_0 = percentage of maximum isometric force. Age=age in months.

Figure 2 EDL Velocity and Power at Heavier Loads Show Increased Age-Related Rate of Decline a) Velocity b) Power Equation: simple linear regression of %reduction in velocity and power of elderly mice (in comparison to adult mice) as a function of load (%P_0). Light loads (10-40%P_0) are represented by diamonds with a dashed regression line and heavy loads (40-90%P_0) are represented by circles and a solid regression line. 40% P_0 is the inflection point indicating where the slope changes and as such the point is included in both sections of the graph. * = slope of line significantly different, $p=0.003$ and $p<0.001$ for velocity and power, respectively.

Figure 3 Force-Velocity Curve Alters with Age: a/P_0 Increases with Age a) EDL b) SOL Equation is a simple linear regression of a/P_0, which describes the shape of the force-velocity curve, with respect to age of the mouse in months. Each symbol represents the a/P_0 of one mouse.

Figure 4 Power Declines with Age a) EDL b) Linear Regression of EDL power at 60%P_0. See **Figure 4** for Pmax and **Figure S3** in the supplement for the other regressions. **c) SOL d) Linear Regression of SOL power at 80%P_0** See **Figure S4** in the supplement for the other regressions. See **Table 2** and Text for post-hoc analysis. Each symbol in **b** and **d** (diamonds) in the regression graphs represents a measurement from an individual mouse at the given age. Equation: simple linear regression of power (y) as a function of age (x). ***** = $p<0.05$, p-value from one-way ANOVA. mN * fl/s= milliNewtons multiplied by fiber lengths per second. $\%P_0$ = percentage of maximum isometric force. Age= age in months.

Figure 5 Further Percent Reduction in Power as $\%P_0$ Increases, Comparing Elderly to Adult. a) EDL There was a -0.26 percent reduction in power per unit increase in load ($\%P_0$, percentage of maximum force at which power was derived)(R=0.993). **b**) **SOL** There was a percentage decrease of -0.10 (R=0.810) in power, per unit increase in in $\%P_0$. Each diamond represents the difference between the mean power production of the entire adult group compared to the entire elderly group). Equation = simple linear regression of percent change in power from adult to elderly (y) as a function of $\%P_0$.

Figure 6 EDL Contraction time to Maximum Force Increases with Age Equation is a simple linear regression of time to maximum force is seconds with respect to age of the mouse in months. Each symbol represents the contraction time of one mouse.

	Power (mN * fl/s)					Velocity (fl/s)				
$\%P_0$	**Adult**	**Old**	**Elderly**	**p-val.**	**%Change**	**Adult**	**Old**	**Elderly**	**p-val.**	**%Change**
					EDL					
0 (Vmax)	**0**	**0**	**0**			**11.0±0.3**	**11.0±0.4**	**10.8±0.3**	**0.921**	**-2**
10	**318±16**	**272±14**	**204±11ab**	**<0.001**	**-33**	**8.2±0.2**	**7.7±0.2**	**7.4±0.2**	**0.064**	**-10**
20	**466±23**	**388±19a**	**289±16ab**	**<0.001**	**-38**	**6.0±0.2**	**5.5±0.1**	**5.2±0.2a**	**0.004**	**-13**
30	**531±26**	**428±21a**	**322±18ab**	**<0.001**	**-39**	**4.6±0.1**	**4.0±0.1a**	**3.9±0.1a**	**0.001**	**-15**
40	**539±29**	**424±23a**	**316±18ab**	**<0.001**	**-41**	**3.5±0.1**	**3.0±0.1a**	**2.8±0.1a**	**0.002**	**-20**
60	**476±26**	**359±25a**	**259±16ab**	**<0.001**	**-46**	**2.1±0.1**	**1.7±0.1a**	**1.6±0.1a**	**<0.001**	**-24**
80	**295±26**	**215±27a**	**139±12ab**	**<0.001**	**-53**	**0.96±0.08**	**0.77±0.09**	**0.63±0.05a**	**0.004**	**-34**
90	**168±21**	**132±22**	**78±10a**	**<0.001**	**-54**	**0.48±0.07**	**0.42±0.06**	**0.32±0.04**	**0.064**	**-33**
100	**0**	**0**	**0**			**0**	**0**	**0**		
P_{max}[§]	**550±27**	**437±17a**	**330±18ab**	**<0.001**	**-40**					
$\underline{\%P_0@p_{max}}$	**38±1.1**	**35±0.9**	**33±0.6a**	**<0.001**	**-13**					
a/P_0	**0.0058**	**0.0084**	**0.012ab**	**<0.001**	**+106**					
					SOL					
0 (V_{max})	**0**	**0**	**0**			**4.6±0.2**	**3.9±0.3**	**3.8±0.2**	**0.097**	**-17**
10	**58±4**	**49±5**	**40±3a**	**0.006**	**-31**	**3.1±0.1**	**2.4±0.1a**	**2.4±0.1a**	**0.001**	**-23**
20	**78±5**	**65±6**	**53±4a**	**0.003**	**-32**	**2.1±0.1**	**1.6±0.1a**	**1.6±0.1a**	**<0.001**	**-24**
30	**82±6**	**69±7**	**58±4a**	**0.006**	**-30**	**1.5±0.1**	**1.2±0.05a**	**1.2±0.07a**	**0.001**	**-20**
40	**81±6**	**69±6a**	**51±5a**	**0.001**	**-38**	**1.1±0.04**	**0.8±0.04a**	**0.8±0.05a**	**0.001**	**-27**
60	**66±5**	**51±4**	**43±3a**	**0.001**	**-35**	**0.6±0.02**	**0.4±0.02a**	**0.4±0.02a**	**<0.001**	**-33**
80	**38±4**	**27±3a**	**24±2a**	**0.001**	**-37**	**0.3±0.04**	**0.2±0.02a**	**0.2±0.01a**	**0.002**	**-33**
90	**23±4**	**15±2**	**14±1a**	**0.020**	**-39**	**0.13±0.02**	**0.09±0.01**	**0.09±0.01**	**0.081**	**-31**
100	**0**	**0**	**0**			**0**	**0**	**0**		
P_{max}[§]	**81±6**	**70±7**	**58±4a**	**0.008**	**-28**					
$\underline{\%P_0@p_{max}}$	**31±0.3**	**29±0.9**	**29±0.3**	**0.266**	**-6**					
a/P_0	**0.021**	**0.024**	**0.027**	**0.067**	**+29**					

Table 1 Power and Velocity at Percentages of Maximum Force ($\%P_0$) V_{max}= maximum unloaded velocity. P_{max} = maximum power. $\%P_0@P_{max}$ = the percentage of maximum force where P_{max} was produced. mN * fl/s = milliNewtons multiplied by fiber lengths per second. P-value was taken from one-way ANOVA. %Change = % change from adult to elderly. Symbols: a=different than adult, b=different than old ($p<.05$) (marked only on old and elderly). [§] = P_{max} values from MatLab data curve-fit, force-power curve derived from the maximum instantaneous velocity (at $\%P_0$) multiplied by force (at the $\%P_0$). In the adult SOL, the derived P_{max} (81±6) is not statistically different than the derived value for power at $30\%P_0$ (82±6).

	Adult	Old	Elderly	p-value
EDL				
mean age	**6.8±0.1**	**23.0±0.4**	**29.9±0.3**	
body	**31.7±0.**	**37.1±1.6**	**32.7±0.7**	**0.006**
mass (mg)	**15.4±3.**	**13.4±1.8**	**11.5±2.3**	**<0.001**
PCSA	**1.23±0.**	**1.02±0.1**	**0.9±0.22**	**<0.001**
P_0 (mN)	**388±17**	**356±16a**	**279±13a,**	**<0.001**
SOL				
mean age	**7.0±0.0**	**23.3±0.5**	**29.9±0.3**	
body	**31.1±0.**	**41.5±3.0**	**32.7±0.7**	**<0.001**
mass (mg)	**14.0±1.**	**12.2±0.4**	**11.9±0.6**	**0.197**
PCSA	**1.16±0.**	**1.04±0.0**	**1.03±0.0**	**0.431**
P_0 (mN)	**187±10**	**195±13**	**166±7**	**0.059**

Table 2 Animal Characteristics for EDL (n=53) and SOL (n=47) Groups Adult mice (EDL n=15, SOL n=12) were used as the reference group to the old (EDL n=14, SOL n=12) and elderly (EDL n=24, SOL=23). P-value was taken from one-way ANOVA (Tukey's HSD post hoc test). PCSA= physiological cross-sectional area of muscle. P_0= maximum tetanic contractile force. Symbols: a=different than adult, b=different than old ($p<0.05$) marked only on old and elderly.

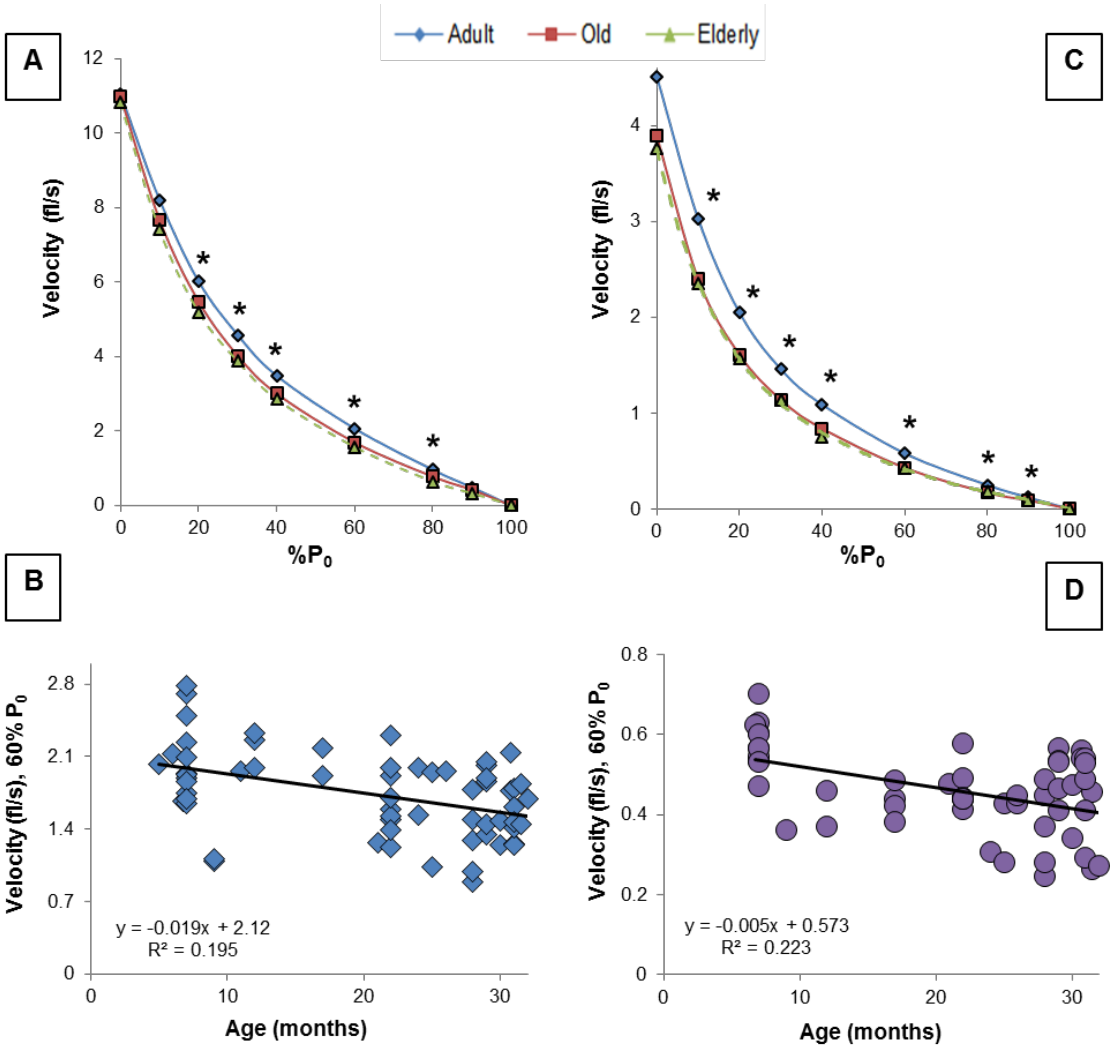


Figure 1 Velocity Declines with Age a) EDL Force-Velocity Curve b) Linear Regression of EDL velocity at 60%P_0 See **Figure S1** in the supplement for the other velocities. **c) SOL Force-Velocity Curve d) Linear Regression of SOL velocity at 60%P_0.** See **Figure S2** in the supplement for the other velocities. See **Table 2** and Text for post-hoc analysis. Each symbol in **b** and **d** (diamonds) in the regression graphs represents a measurement from an individual mouse at the given age. Equation: simple linear regression of velocity (y) as a function of age (x). ***** = $p<0.05$; p-value from One-Way ANOVA. fl/s= fiber length per second. %P_0 = percentage of maximum isometric force. Age=age in months.

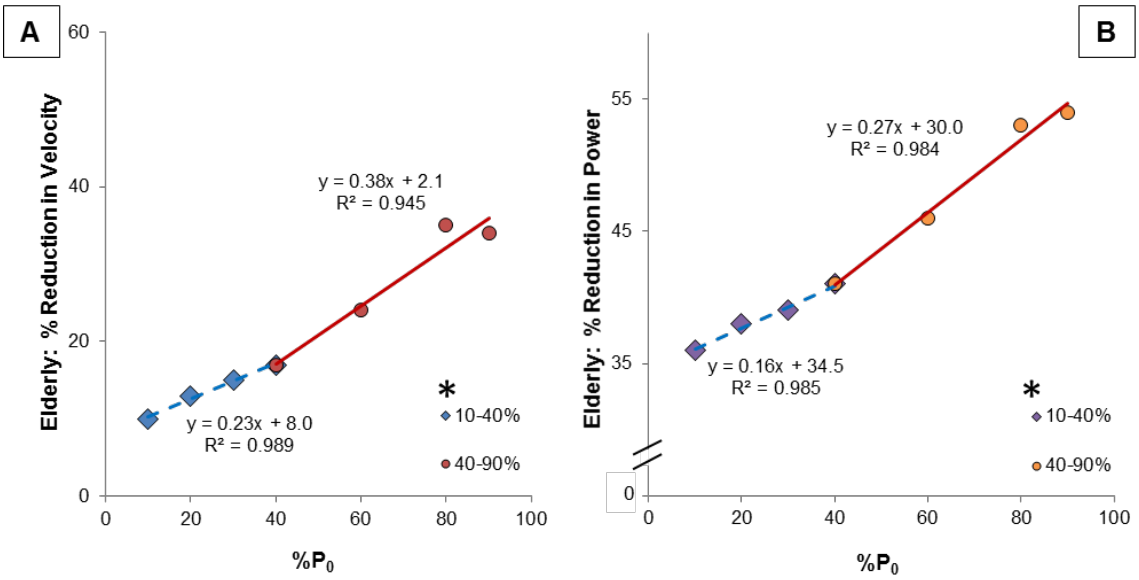
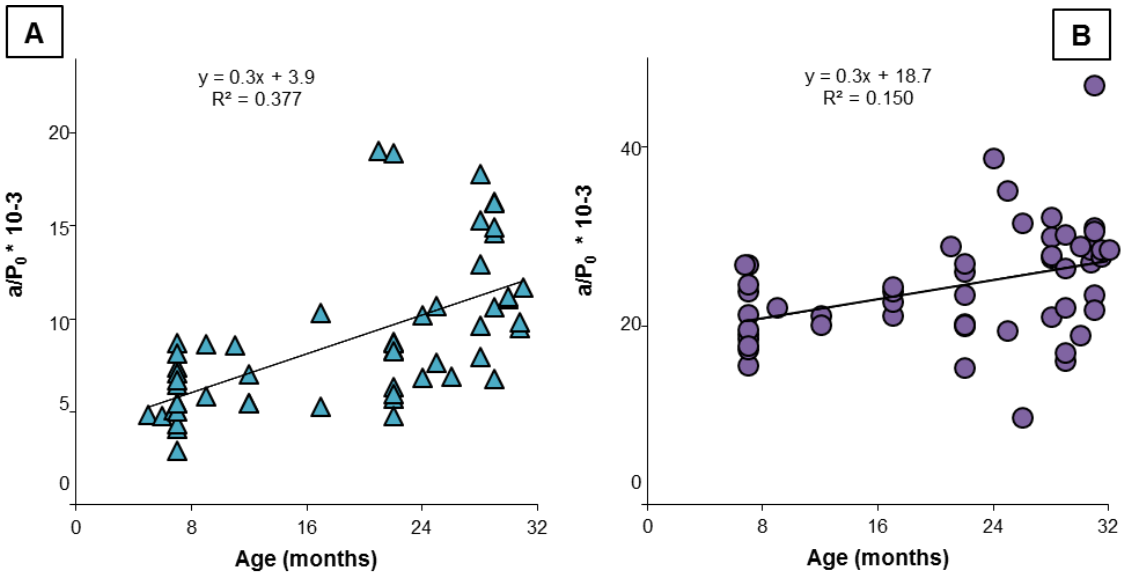


Figure 2 EDL Velocity and Power at Heavier Loads Show Increased Age-Related Rate of Decline a) Velocity b) Power Equation: simple linear regression of %reduction in velocity and power of elderly mice (in comparison to adult mice) as a function of load (%P_0). Light loads (10-40%P_0) are represented by diamonds with a dashed regression line and heavy loads (40-90%P_0) are represented by circles and a solid regression line. 40% P_0 is the inflection point indicating where the slope changes and as such the point is included in both sections of the graph. * = slope of line significantly different, p=0.003 and p<0.001 for velocity and power, respectively.



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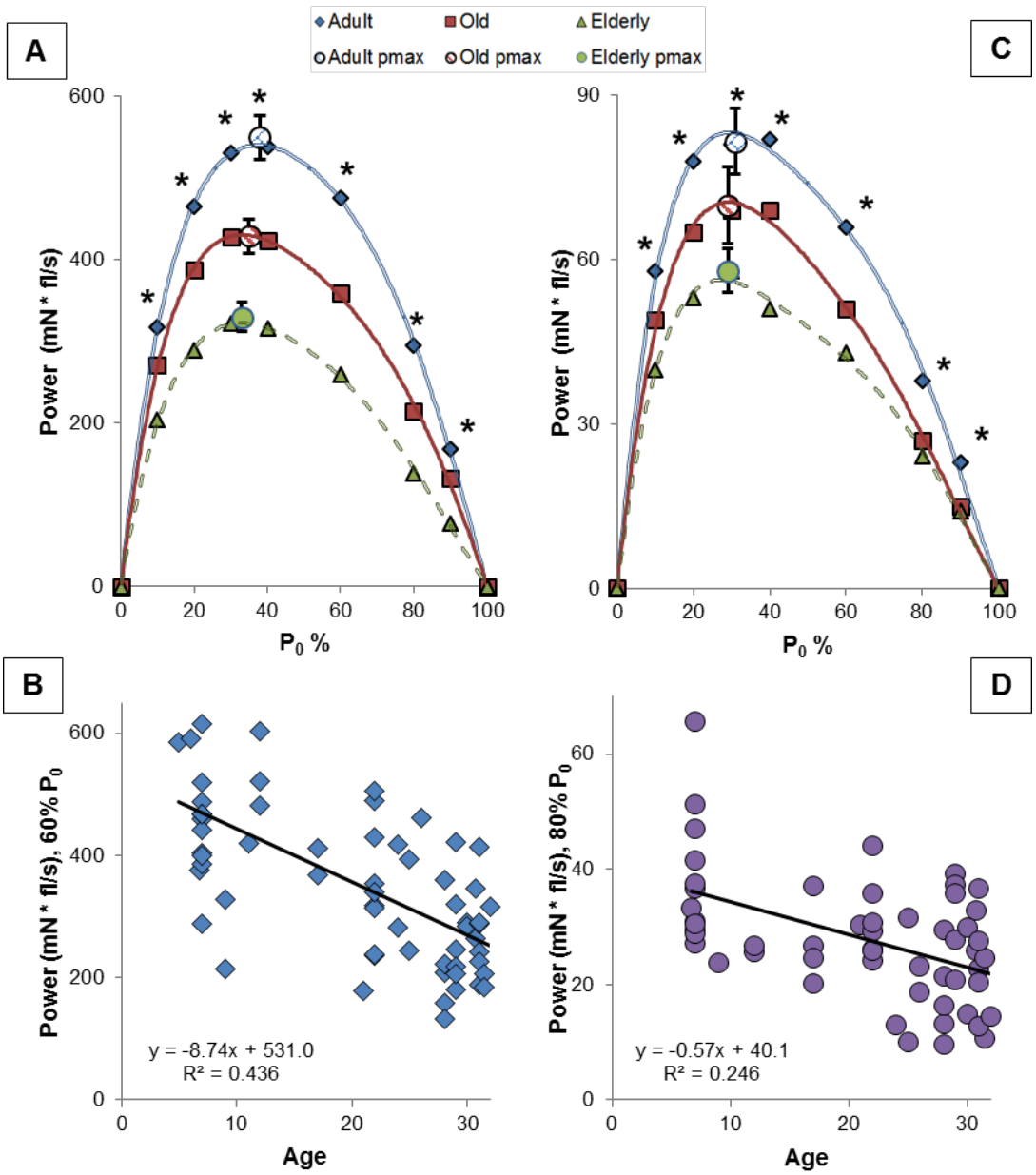

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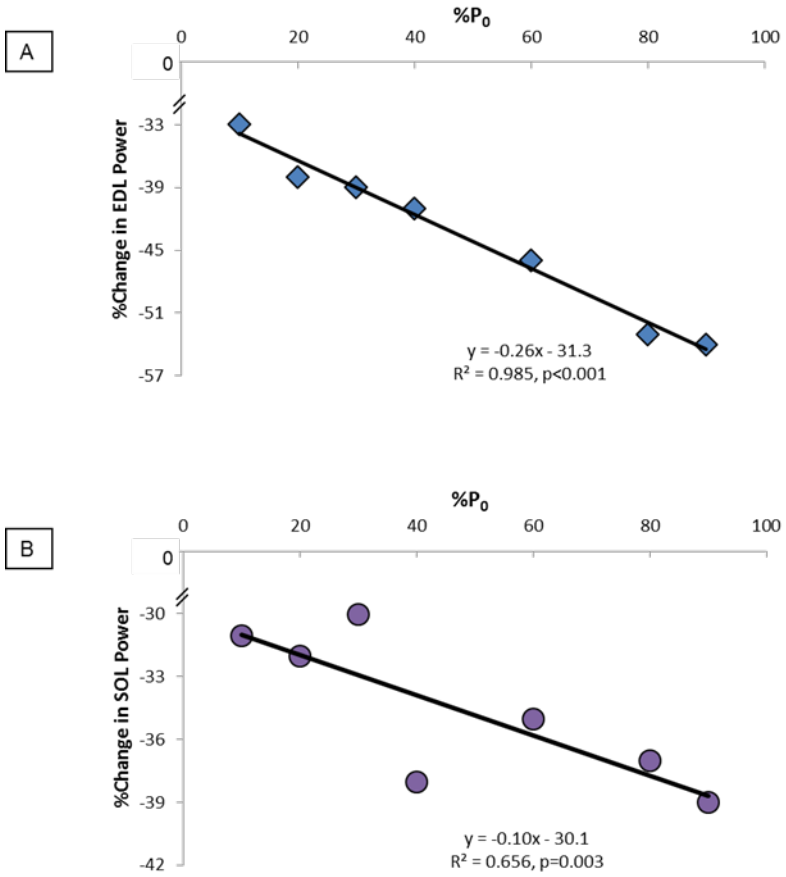

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Figure 6

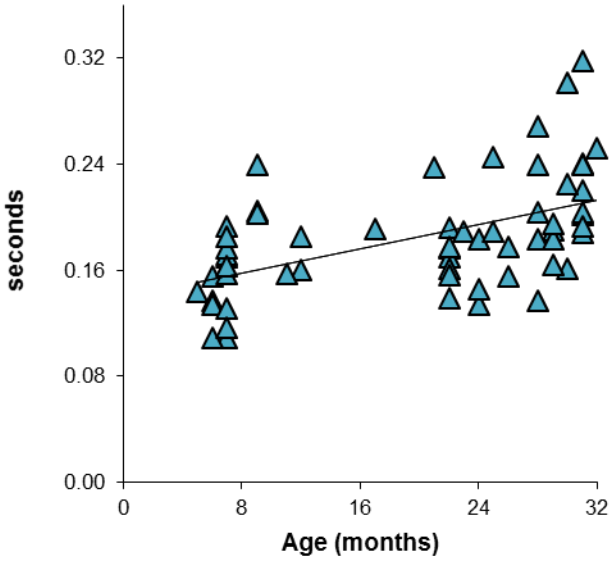

Figure 6 EDL Contraction time to Maximum Force Increases with Age Equation is a simple linear regression of time to maximum force is seconds with respect to age of the mouse in months. Each symbol represents the contraction time of one mouse.

Online Resource 1

APPENDIX: Relationships between Force, Velocity, and Maximum Power

In order to evaluate the relationship between velocity, force and maximum power, we examined simple and multiple linear regressions to determine how much variability is explained by each component of power [P_0, velocity, and incorporating $\%P_0@P_{max}$ (the percentage of maximum force where the muscle produced maximum power) and age to hierarchical multiple regression analysis]. In the EDL, P_0 explained 58% of the individual variability in P_{max} (P_0 + age, 66%). At $30\%P_0$ the velocity of contraction explained 23% of P_{max} (velocity + age, 49%), and when the two components (P_0 and velocity at 30% P_0) were combined 73% of P_{max} variability was explained. Adding a third component, age, 75% of the variability in P_{max} was explained. In the SOL, the P_0 explains 59% of the individual variability (P_0 + age, not significantly different). At $20\%P_0$ the velocity of contraction explains 50% of the variability (velocity + age, no difference), and when combining the two components, 83% is explained (adding age yields 84% of the variability). (**Online Resource 2**, **Figure S5**)

For simplicities sake, we will use the convention of V30 to represent velocity (v) as measured at 30% of P_0 (30). Simple linear regression demonstrated a strong positive correlation, as would be expected, of EDL P_{max} with P_0 [R=0.79, r^2=0.630, p-value<.001; equation: Pmax (mN*fl/s) = -33.48 + 1.36 * P_0]; as well as with contractile velocity at $30\%P_0$ [R=0.51, r^2=0.260, p<.001; equation: P_{max}

(mN*fl/s) = -0.90 + 102.43 * v30]. (**Online Resource 2, Figure S5**) We performed a factor analysis (promax rotation) to determine which of the collected outcome variables contributed most to the variability in the system (overall model). The results of our factor analysis yielded that P_0, v30, v40 and v60 were the main sources of variability in P_{max}. Interestingly, the only velocity that was not significantly correlated with the EDL P_{max} was V_{max}.

After analyzing the velocities separately (**Online Resource 2, Figure S1**), v30 was selected to be used in the multiple regression analysis and the combination of P_0 and v30 explained 79% of the variability in the observed P_{max} (R=0.853, r^2=0.728, p<.001; equation: P_{max} (mN*fl/s) = -316.33 + 1.22 * P_0 + 77.8 * v30]). $\%P_0@P_{max}$ was correlated with P_{max} (R=0.398, r^2=0.159, p=.001), but did not significantly add information when combined with the above multiple regression (R=0.891). When age was incorporated into the model (multiple regression of P_{max} with P_0, V30 and age of mouse); however, the r^2 was increased to 0.749 (coefficient significance p=.032). $\%P_0@P_{max}$ was correlated with P_{max} (R=0.398, r^2=0.159, p=0.001), but did not significantly add information when combined to the multiple regression.

P_{max} in the SOL followed a similar pattern. Regression of P_{max} with P_0 (R=0.762, r2=.577, p<.001; equation: P_{max} (mN*fl/s) = -17.65 + 0.46 * P_0]; as well as with contractile velocity at v20 [R=0.714, r^2=0.501, p<.001; equation: P_{max} (mN*fl/s) = -

13.78 + 46.78 * v20] showed strong correlations. A principle component factor analysis revealed that the top 4 factors, in descending order of R-values (in parenthesis), correlating to the P_{max} in the SOL were: P_0 (0.762), v20 (0.714), V_{max} (0.71) and v30 (0.637). The most explanatory/predictive equation was derived from a regression of P_{max} with P_0 and v20 [R=0.915, r^2=0.837, p<.001; equation: P_{max} (mN*fl/s) = -58.412 + 0.359 * P_0 + 34.842 * v30]. In the SOL, the %P_0@P_{max} was not significantly correlated with P_{max}.

Online Resource 2
Supplemental Figures S1-S7

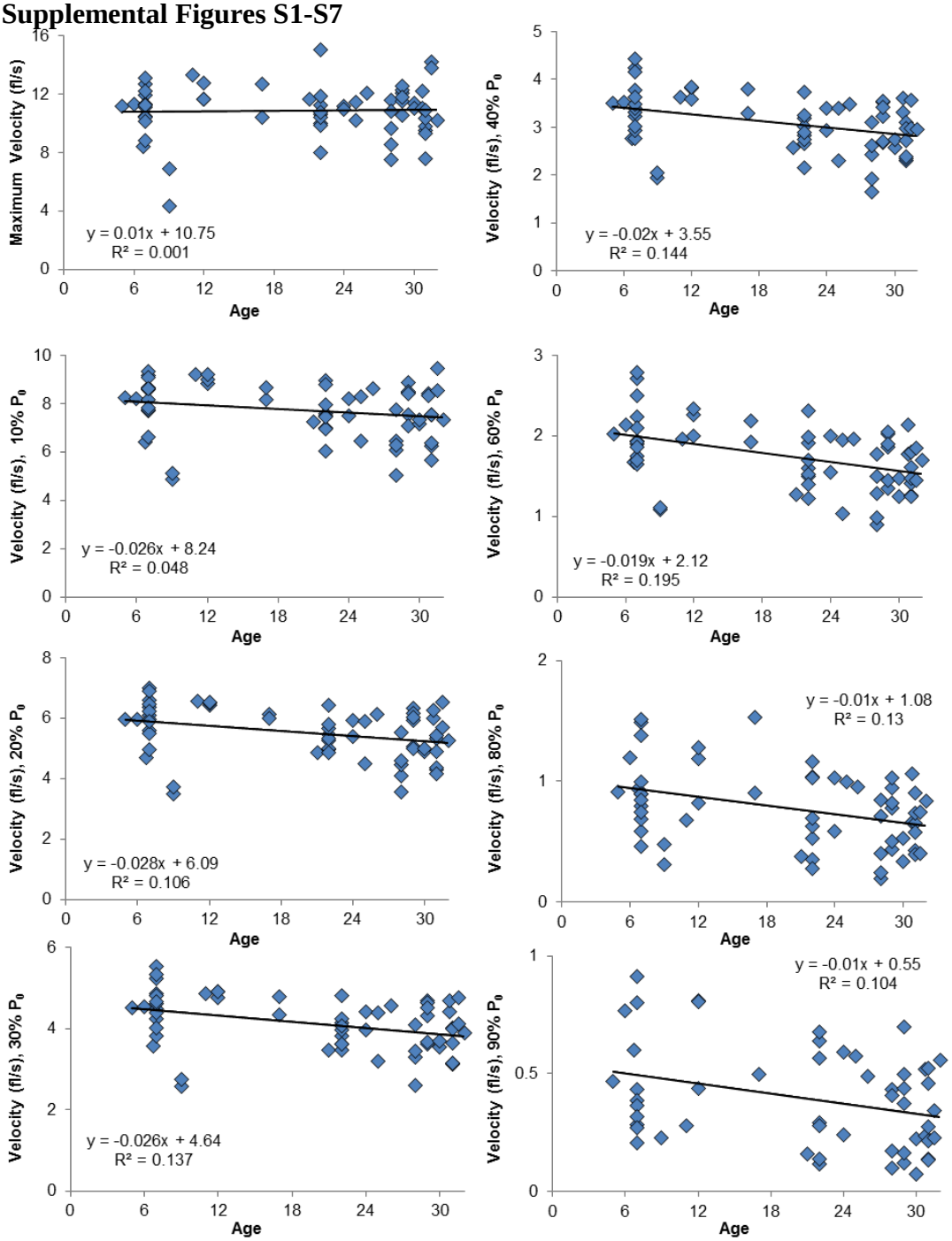

Figure S1 EDL Loaded Velocity Declined with Age. There was a significant negative correlation between velocity and age at the measured loads (20-90%P_0). V_{max}, however, (which was non-physiological and represented what velocity would be if a muscle was completely unloaded) did not change in a linear manner over the lifespan. Each diamond represents a measurement from an individual mouse. Age: Age of mouse in months. Equation: simple linear regression of velocity (y) as a function of age (x). fl/s = fiber lengths per second. x% P_0 = percentage of maximum tetanic force when the velocity measurement was taken. Maximum Velocity: V_{max}, maximum unloaded velocity.

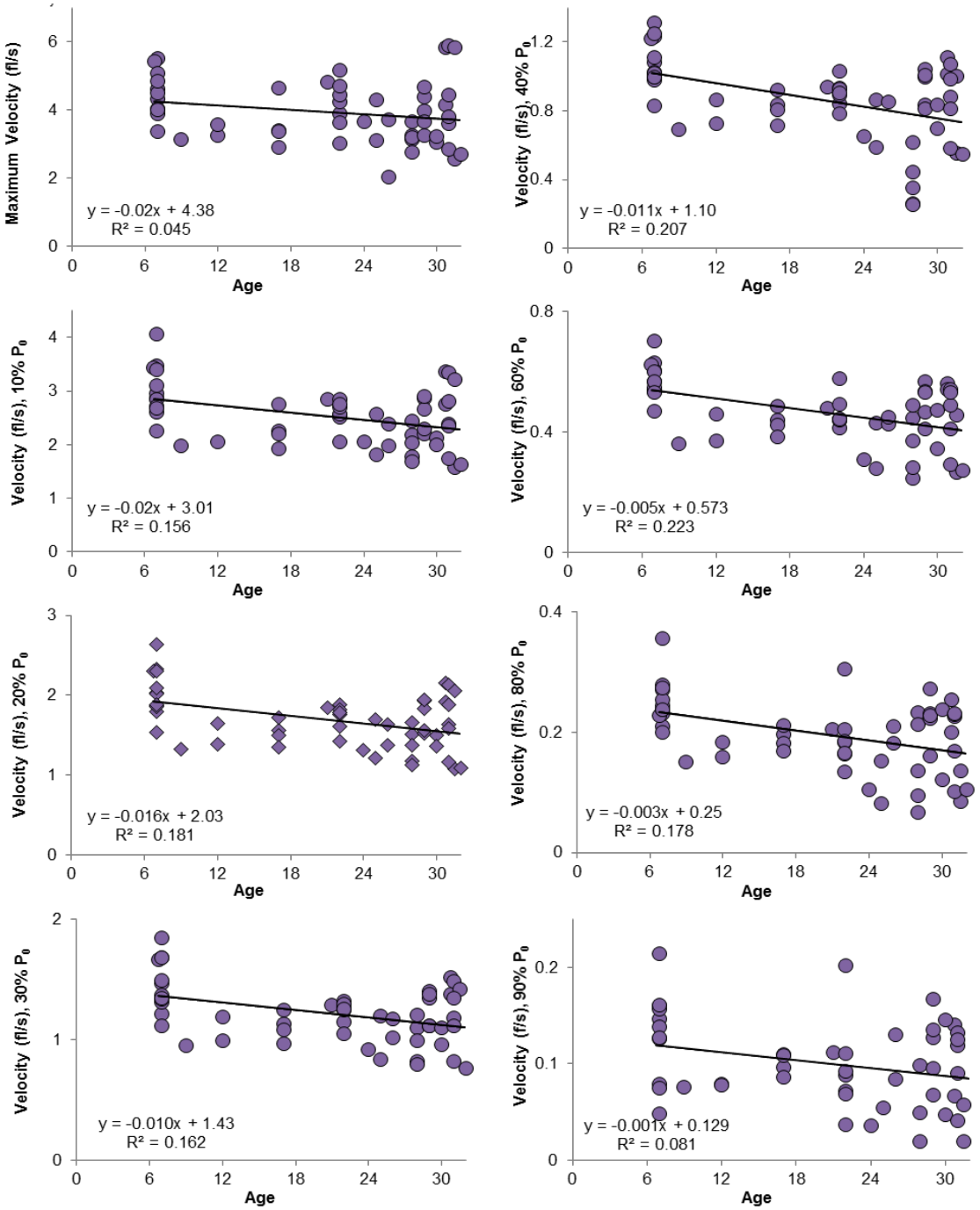

Figure S2 SOL Loaded Velocity Declined with Age. There was a significant negative correlation between velocity and age at the measured loads (10-90%P_0). V_{max} (which was non-physiological and represented what velocity would be if a muscle was completely unloaded) did not change in a significant linear manner over the lifespan. Each diamond represents a measurement from an individual mouse. Age: Age of mouse in months. Equation: simple linear regression of velocity (y) as a function of age (x). fl/s = fiber lengths per second. x% P_0 = percentage of maximum tetanic force when the velocity measurement was taken. Maximum Velocity: V_{max}, maximum unloaded velocity.

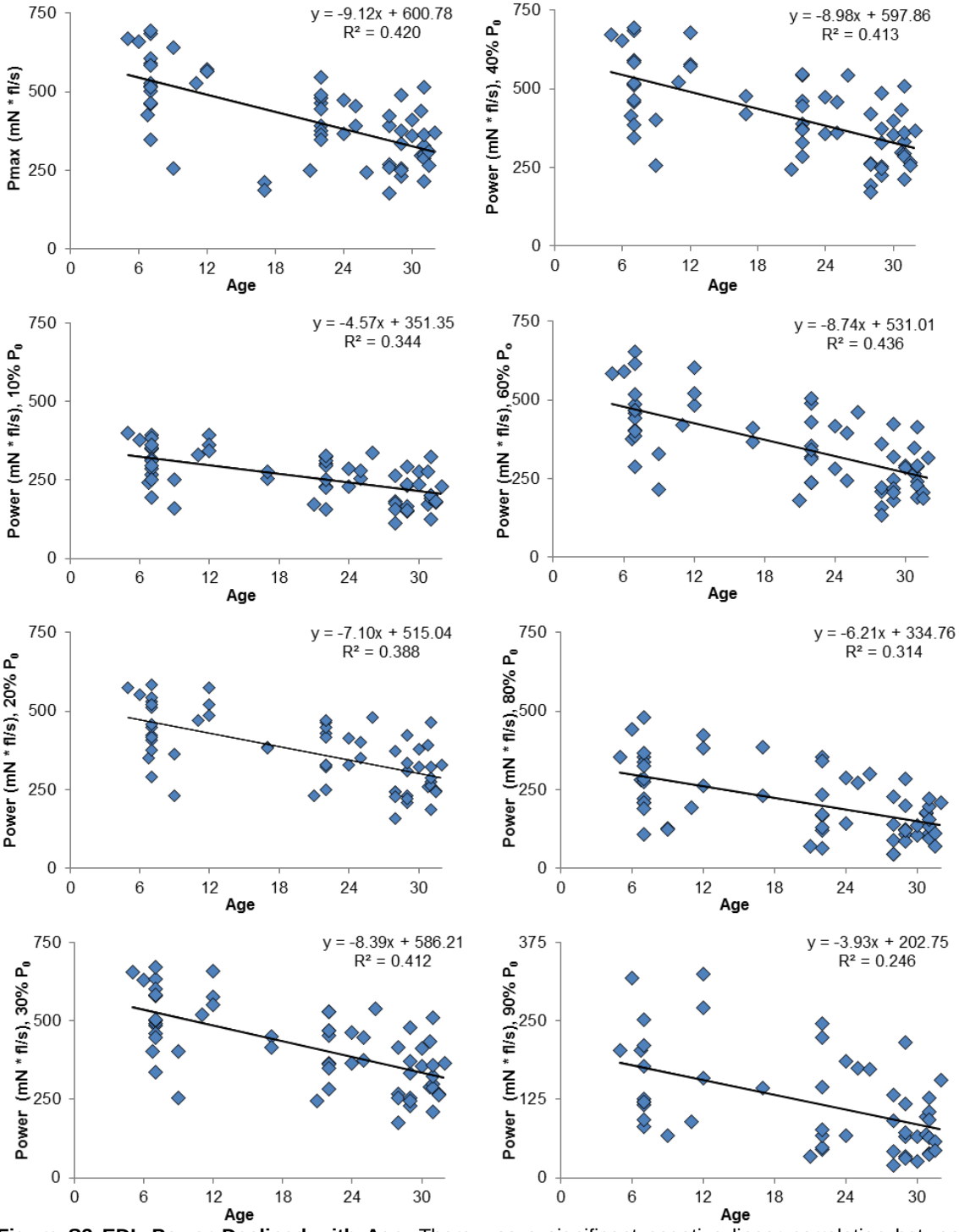

Figure S3 EDL Power Declined with Age. There was a significant negative linear correlation between power production and age at the measured loads (10-90%P_0) and at P_{max} (maximum power). x% P_0= "x" percentage of maximum tetanic force when the power measurement was derived. Each diamond represents a measurement from an individual mouse. Power measured in milliNewtons * fiber lengths per second (mN * fl/s). Age: age of mouse in months. Equation: simple linear regression of power (y) as a function of age (x).

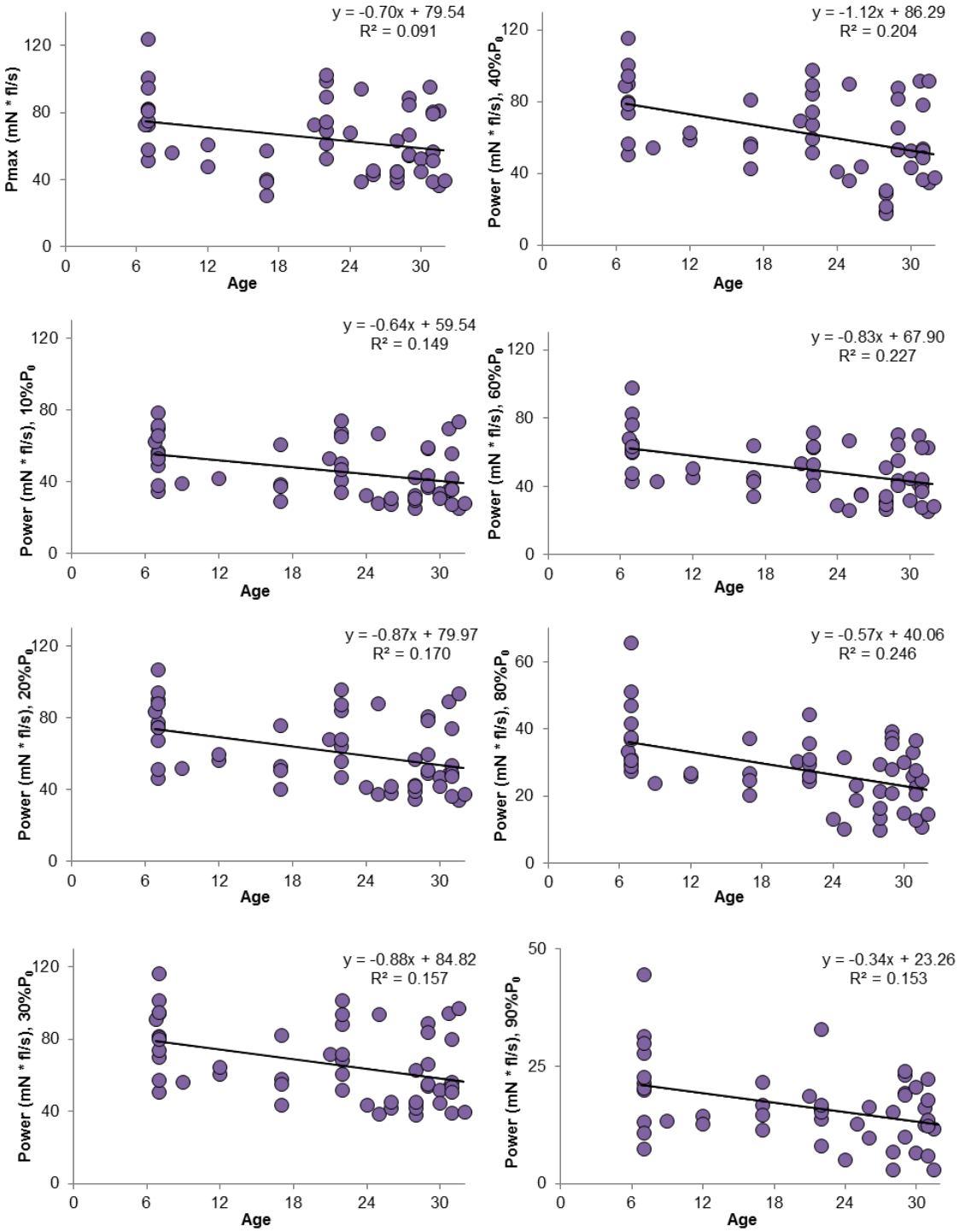

Figure S4 SOL Power Production Declined with Age. There was a significant negative correlation between power and age at the measured loads (10-90%P_0) and at P_{max}. Power measured in milliNewtons * fiber lengths per sec (mN*fl/sec). x% P_0= "x" percentage of maximum tetanic force when the power measurement was derived. Each diamond represents a measurement from an individual mouse. Age: age of mouse in months. Equation: simple linear regression of power (y) as a function of age (x).

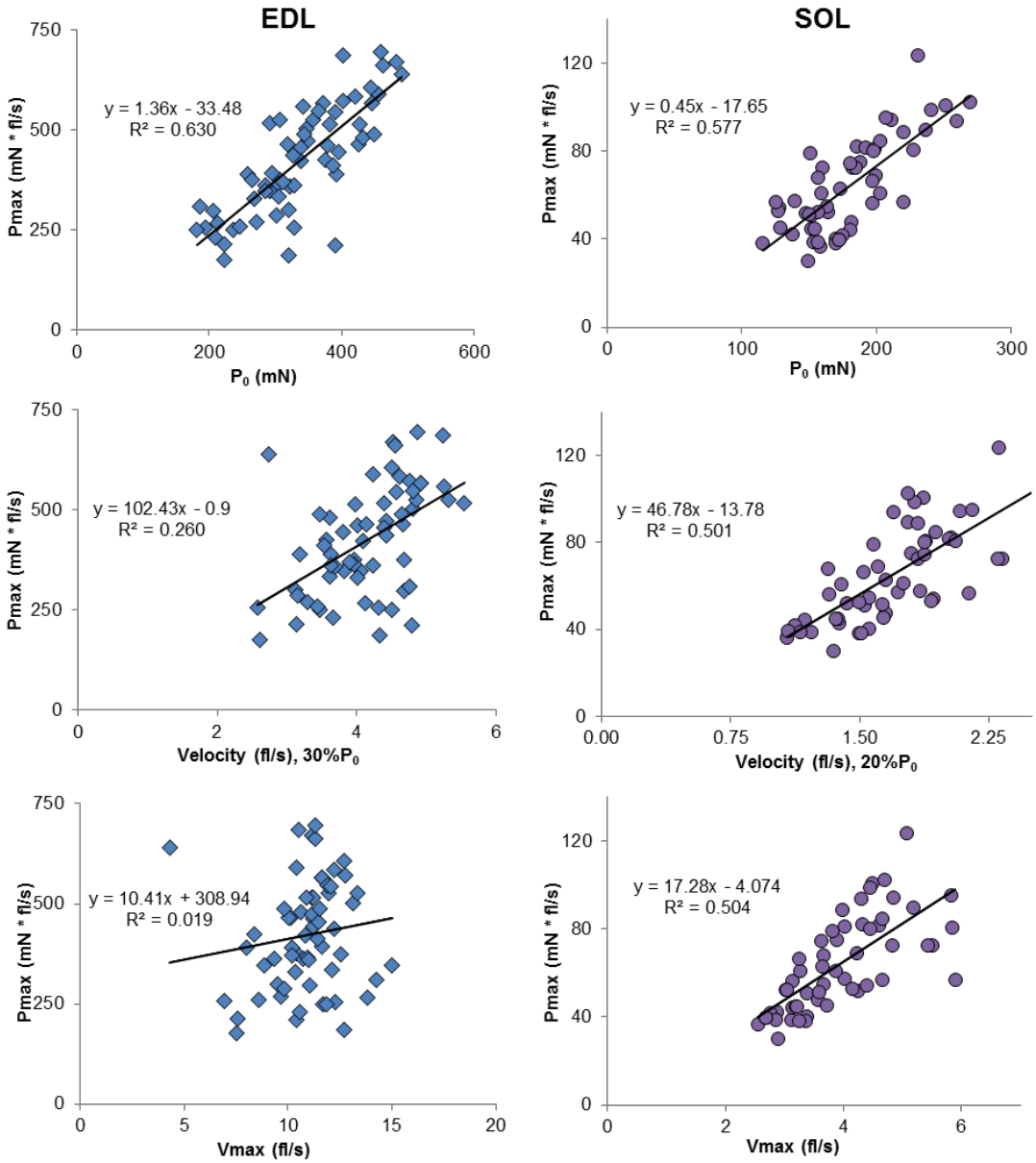

Figure S5 Relationship of Pmax, P_0 and Velocity. In both the EDL and SOL, P_{max} (maximum power) and P_0 (maximum tetanic force) (R=0.79 and 0.76, respectively, both p<0.001), and P_{max} and Velocity (R=0.51 and 0.71, both p<0.001) were correlated. In the EDL, P_{max} and V_{max} (maximum unloaded velocity) were not related. However, there was a significant correlation (R=0.71) between SOL V_{max} and P_{max}. P_{max} measured in milliNewtons * fiber lengths per second (mN * fl/s) and velocity measured in fiber lengths per second (fl/s). Each symbol (diamond for EDL and circle for SOL) represents measurements taken from one mouse. Equation is of a simple linear regression of P_{max} (y) being a function of the x-axis variable (P_0, velocity x% of P_0, or V_{max}). x% P_0 = "x" percentage of maximum force when measurement was taken.

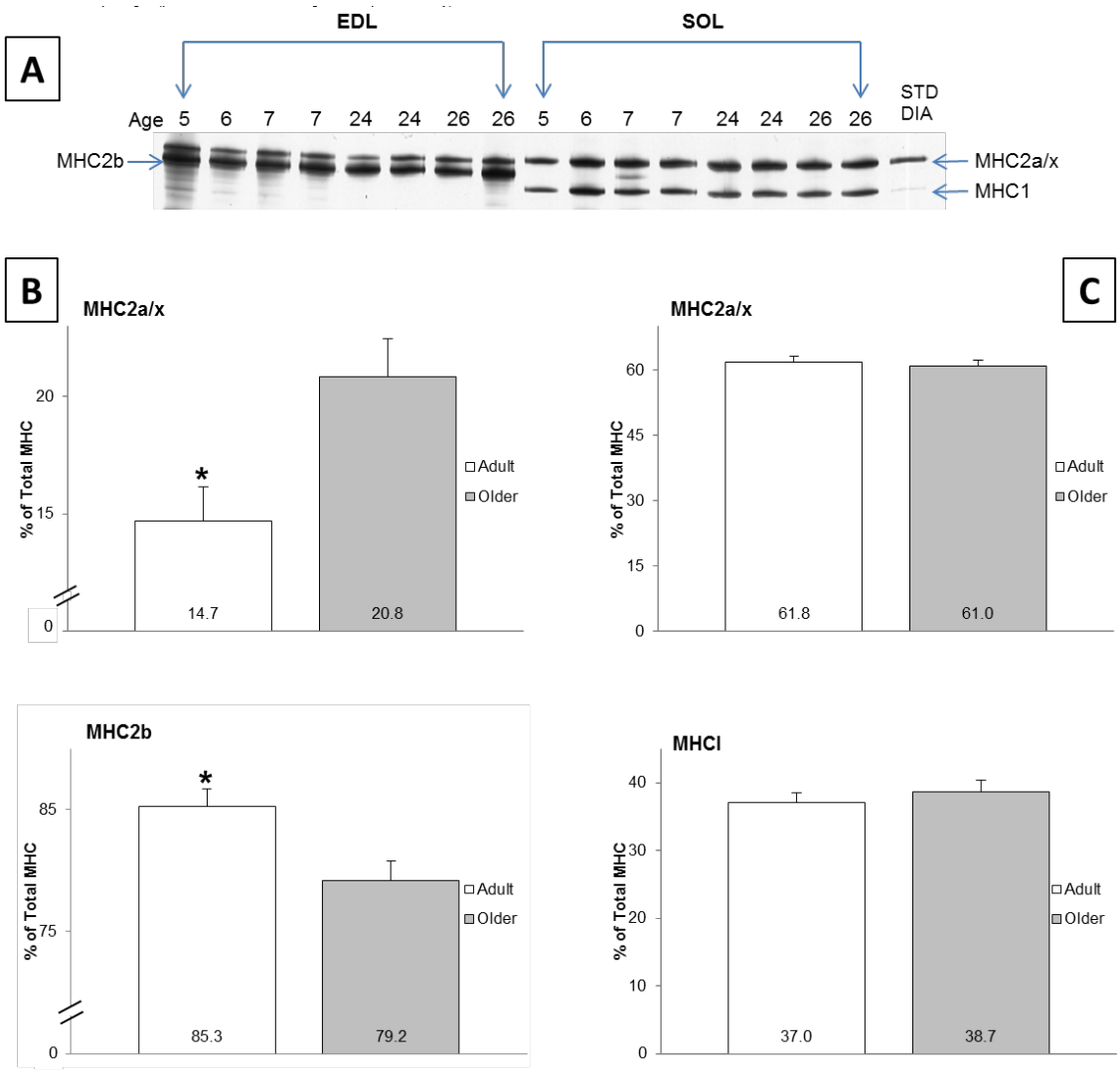

Figure S6 MHC Composition with Age. A. Representative Gel Image. Silverstained 5% Acrylamide large format gel. **B. EDL** Expression of MHC2/ax increased by 6% in older (21%, n=25, mean age=28 months, p=0.009) compared to adult mice (15%, n=18, mean age=7 months) using Student's t-test, implying a shift towards a slower isoform composition. Conversely, MHC2b expression is reduced by 6% with age (Adult 85%, Old 79%, p=0.01). **C. SOL** There was no change in the SOL MHC expression profile (Adult: MHC2ax 62%, MHC1 37%, n=15, mean age=7.8 months; Older: MHC2ax 61%, MHC1 39%, n=18, mean age=27.8). Each lane represents whole homogenized muscle of one mouse at the age listed on top. Age: age in months of mouse. STD DIA: standard from rat diaphragm (proteins identified using mass spectrometry).

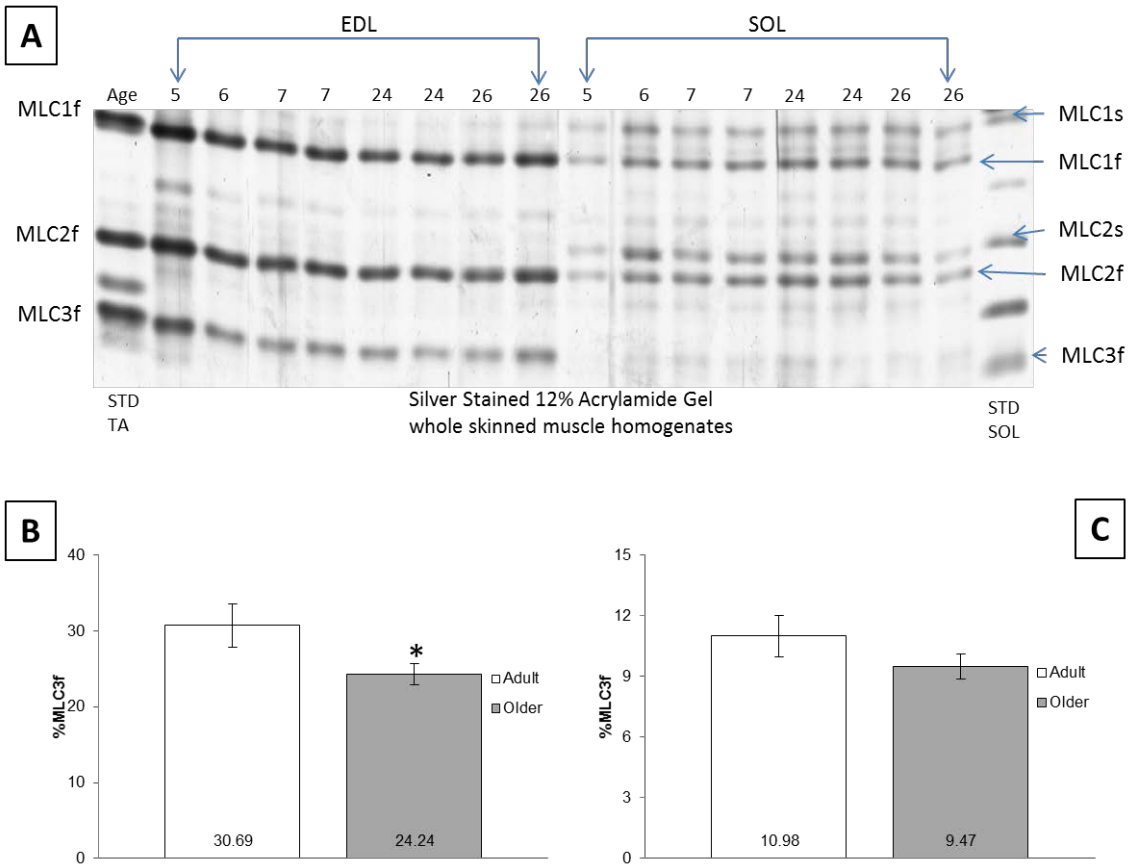

Figure S7 MLC3f Percentage Decreased 21% with Age in EDL. A. Representative Gel Image. Silver-stained 12% Acrylamide large format gel. **B. EDL** There was a 21% decline in MLC3f with age**. C. SOL** There was no change in MLC3f. Adult group (EDL, 31% MLC3f, n=19, mean age=7.5; SOL % MLC3f, n=18, mean age=7.4) was compared to combined old/elderly group, Older (EDL, 24% MLC3f, n=23, mean age =27.9; SOL, % MLC3f, n=22, mean age=27.8) using Student's t-test (EDL, p=0.03; SOL p=0.20). Each lane represents whole homogenized permeabalized muscle of one mouse at the age listed on top. Age= age in months of mouse. STD TA: rat tibialis anterior standard. STD SOL: rat SOL standard. Standard proteins identified via mass spectrometry. MLC (myosin light chain) is shown with three essential light chain isoforms (1s, 1f, 3f) and 2 two regulatory light chain isoforms (2s, 2f). s = slow. f = fast .Number in base of columns in chart equals average percentage of MLC3f out of total. Note: %MLC3f is out of the total of fast MLC [MLC3f / (MLC1f+MLC3f)], in SOL MLC1s was not counted.

Interlude 2 Resistance Training to Improve Neuromotor and Contractile Function

Age-related muscle dysfunction is evident in both humans and mice (Chapters 1-3). Specifically in Chapters 2 and 3, with a comprehensive analysis of functional performance and muscle contractility we established an evident age-related decline in ability in the C57BL6 mouse. For the first time, age-related contractile dysfunction was demonstrated to manifest to a greater extent when the muscles were contracting against heavier load (at higher percentages of P_0) then at lighter loads.

Resistance training is an accepted treatment for sarcopenia, resulting in the improvement of strength, muscle mass and functional ability (explained in detail in Chapter 1). To the best of our knowledge, there is no model of voluntary resistance training for mice based upon human principles of weight lifting. We therefore provided the scientific community with *a mouse model mimetic of human weight training*, as would be performed in the gymnasium.

Chapter 4 highlights this voluntary resistance training protocol for the mouse. We selected a comprehensive set of outcome measures. The tests were designed to determine if the mice would adapt as humans would if undergoing a similar exercise program. If the preponderance of evidence pointed to these positive

adaptations being made, we considered the protocol to be a valid mimetic of weight training. The validated model will be valuable for pre-clinical investigation of the synergistic effects of other treatments (e.g. pharmaceutical, nutritional, hormonal) with resistance training, and for mechanistic investigations of basic exercise biology.

Chapter 4

This is a pre-copy-editing, author-produced PDF of an article submitted for publication following peer review.

Voluntary Resistance Training in Adult Mice

Ted G Graber[1], Katie R Fandrey[2], LaDora V Thompson[3]
Department of Physical Medicine and Rehabilitation
University of Minnesota Medical School

TGG primary author of manuscript and study designer, conducted all animal training, performed assays, collected and analyzed primary data, email: grab0170@umn.edu

KRF collated and recorded training physiology data, manuscript review, email: pitts026@umn.edu

LVT guarantor of study, assisted in study design and data interpretation, major contribution to manuscript writing, scientific manuscript review, funding, email: thomp067@umn.edu

Running Head: Resistance Training Protocol for Mice

Corresponding Author: LaDora V. Thompson
Email: thomp067@umn.edu, Phone: 612-626-5271 (office), 612-624-2436 (fax)
University of Minnesota Medical School Department of Physical Medicine and Rehabilitation
Program in Physical Therapy
Rm 366A ChRC
426 Church Street SE
Minneapolis, MN 55455

Abstract

The current hypertrophy models used to investigate cellular mechanisms in mice are less representative of voluntary human weight (resistance) training. Thus, the main purpose of this study was to establish a mouse model that mimics human weight training. This model has wide application to study not only exercise biology, but also to investigate the efficacy and mechanisms of resistance exercise as an intervention for conditions such as sarcopenia.

Male C57BL/6 mice (12 months of age at study conclusion) were randomly separated into control (n=9) and trained groups (n=6). The trained group used progressive resistance (via a weight harness) and intensity (~4-10 RPM) on a custom motorized running wheel (possible speed from 1-10 RPM). The mice trained on a voluntary program similar to a human beginning workout regimen (4-5 sets/session, 3 sessions/week, for 12 weeks).

The trained mice demonstrated muscle hypertrophy (soleus mass and plantaris muscle fiber area), increased soleus force/power production (*in vitro* contractile physiology), and improvement in markers of training physiology (force and power output per session) and overall neuromuscular function (rotarod). The model produced results in the mice similar to what would be expected after humans engaged in a period of weight training, thus, this validated protocol has the potential to serve as a valuable tool for future pre-clinical and mechanistic investigations.

Keywords: Resistance Training, Mice, Exercise Physiology, Muscle

INTRODUCTION

In order to evaluate the efficacy and safety of novel interventions and their synergistic effects with weight training prior to clinical testing in humans, an animal model of voluntary resistance training is needed. While some models of resistance training do exist in the rat such as: a squat rack mimic device, first characterized by Tamaki and colleagues (Tamaki, 1992; Krisan, 2004; Drummond, 2009), a weighted backpack and standing model (Farrell, 1999; Flukey, 1995) or the tail weight ladder climb (Deschenes, 2000), they are not fully voluntary as participation is induced via operant condition (punishment avoidance) in the squat and backpack models with an electric shock to either the tail or the feet, respectively, or in the ladder climb with a spray of cold water. Other research has used food reward as an alternative to punishment for conditioning, but in these models the rats must be food restricted in order to induce hunger (Wirth, 2003), which involves its own external stressors. Therefore, the need exists for a mouse model that closely approximates human weight-lifting exercise, is voluntary, and follows physiological principles.

To our knowledge, no representative mouse models of voluntary and physiological progressive weight training exist. However, there are models of hypertrophy (Always, 2005; Cholewa, 2014), such as synergistic ablation (Timson, 1985) or electrical stimulation (Ryan, 2010). Available mouse models of

hypertrophy are not fully ‘translational’ as models of voluntary resistance training because an optimal translational model follows principles of biomechanics, nerve and muscle physiology, and mimics voluntary human training.

The C57BL/6 inbred mouse model is used extensively in research. The mouse has numerous advantages as a model organism including: *1)* being a tractable model (Martin, 2011) with numerous transgenic models readily available for study, as well as the ability to produce novel genetic knock-outs and knock-ins (Yuan, 2011; Wurst, 2006), *2)* having a relatively shorter lifespan (~3 years) that allows improved capacity to perform longitudinal aging studies, *3)* a much lower price point in comparison to other mammalian models, both in initial cost and in per diem housing and husbandry (therefore allowing for a larger number of subjects per study, increasing the robustness of the results), and 4) are close genetically to humans (99% shared genome) (Bobuski, 2002).

The main purpose of this study was to produce a mouse exercise protocol that mimics human weight training as would be performed in the gymnasium. We hypothesized that the effect of this training protocol would be to improve the physiology/morphology of the mice similarly to how a human would be improved after undergoing a training study based upon the same training principles. To test this hypothesis, we developed and constructed custom equipment (**Figure 1)**, designed the protocol based upon basic human principles of weight training (see

Table 1 and Figure 2), and selected the following outcome measurements to assess adaptation: muscle hypertrophy (soleus muscle wet mass and plantaris muscle cell size), soleus muscle contractility (*in vitro* force, velocity and power), measurements of training performance adaptations (force, work and power output per training session, and correlations with muscle cell size), and animal performance (rotarod and inverted cling grip test). The successful validated protocol will serve in future investigations as a pre-clinical model of voluntary resistance training intervention.

Important areas of research that will benefit from a mouse model of voluntary resistance training are aging muscle biology, sarcopenia, and frailty (Marzetti, 2006; Sundell, 2011; Rolland, 2008; Breen, 2011; Janssen, 2002). Progressive resistance training, weight lifting as the gold standard, is an accepted treatment that slows the progression of sarcopenia (Marini, 2008; Fiaterone, 1990; Pillard, 2011). In humans of any age resistance exercise reduces age-related muscle fiber apoptosis (Marzetti, 2006), induces gains in strength and muscle mass (Marzetti, 2006), improves functional outcome measurements and confers positive metabolic changes (Marini, 2008; Capodaglio, 2007; Fiaterone, 1990). While the strategy of resistance training has benefits, it does not totally reverse sarcopenia. Research has been conducted in other effective strategies such as protein or branch chain amino acid supplementation (Churchward-Venne, 2012; Dickinson, 2014; Walker, 2011).Therefore, it is likely that effective treatments for

sarcopenia in the 21st century will involve the synergy of multiple interventions (e.g., exercise, nutrition, pharmaceutical, and ergogenic) (Breen, 2011; Candow, 2011, Walrand, 2011). The mouse model of resistance training will be a valuable resource for researchers to investigate both mechanisms of exercise response and to evaluate the efficacy of nutritional supplements, pharmaceuticals and ergogenic aids in conjunction with resistance training (Cholewa, 2014).

METHODOLOGY

Animal Model

C57BL/6 male mice from the NIA Aging Colony (adults, 12 months old=100% survival) were randomly selected to control (n=9) or exercise groups (n=6) and treated in accordance with approved IACUC protocol. The mice were group housed with 12-hour light cycle at 22°C and fed *ad libitum*. Mice were weighed (massed) before and after the training period and BMI (body mass index) was calculated as mass (g) / length (cm).

Training Equipment and Protocol

The main purpose of the study was to validate a resistance training protocol for mice that mimicked human weight training as would be performed in a gymnasium, specifically the effect of human weightlifting exercises such as the "farmer's walk" or the "walking lunge". Two critical pieces of equipment were custom-designed and fabricated (**Figure 1**). The first was a powered running wheel engineered by mounting a steel caged-in 11-inch running wheel to a rotary electric motor with a speed controller capable of rotating from 1-10 revolutions per minute (rpm). The second indispensable piece of equipment was a small Velcro weight harness to which small weights were applied. The mice adapted to the weighted harness and the powered running wheel. Principles of weight training were incorporated into the program: progressive resistance/intensity (ACSM, 2009; Kraemer, 2004), training frequency (ACSM, 2009), number of sets

per day (ACSM, 2009, Krieger, 2009), warm-up sets (ACSM, 2009), set duration (Toigo, 2006), and rest between sets (ACSM, 2009; de Salles, 2009). The program was designed to be similar in scope to a typical human weight training study or exercise program for beginners (ACSM, 2009; de Salles, 2009; Bird, 2005; Kraemer, 2004; Schoenfeld, 2010; Schoenfeld, 2011). Principles involved in designing the training protocol for the mice are found in **Table 1**.

Figure 2 describes the overall research design and time course in detail. In week one the mice underwent baseline testing for functional performance (rotarod and grip test). Following functional testing, the mice went through two different training periods, *1)* acclimation training and *2)* weight training. Acclimation training introduces both the weight harness and running wheel, and the mice are trained to use the equipment.

Acclimation training

In the first session of acclimation training, the mice underwent “scruff” training (scruffing being the standard method of picking up and controlling a mouse by firmly grasping the skin around the neck/front shoulders) where the mice were individually “scruffed” (held for approximately 5), 3 times in succession (about 30 seconds between trials). The goal was to allow the mice to become accustomed to being handled and scruffed, because in the beginning of training mice often needed to be scruffed to administer the weight harness.

In the second session, the mice were introduced to the running wheel. The running wheel was set at a low speed (4rpm) and they quickly learned to walk on the wheel. Note, mice seem to innately/instinctively understand the running wheel concept and it is not difficult for them to master, even mice encountering a wheel in the wild will use one (40). Next, during the following training session, the mice were trained to accept the weight harness. In order to have a mouse accept the weight harness successfully, a modified single banded harness with no weights was placed over their shoulders and then removed after a short period (~5sec). This step is repeated 3 times during the training session.

In the next training session, the harness (without weights) was placed on the mouse and the mouse was put into the powered running wheel (4 rpm) for 30 seconds. Once again, this step was repeated 3 times. On the last day of acclimation training the mice wore the harness with a light weight (3.2g) and were then placed in the wheel for 3 repetitions of 30 seconds at 4 rpm. This last day of acclimation training was also the first session of the weight training described below.

Weight Training

In the weight training, the mice initially worked for the first week with the very light weight (3.2g). After the third day of using 3.2g, the mice were introduced to the

double-banded harness (**Figure 1A**) that was used throughout the remainder of the training. During this session the weight in the harness was increased to 4.5 grams. At this stage of the training, the weights placed in the harness increased 10-15% every 3rd to 5th session (depending upon the progression of the mice cohort) with the goal of carrying ~50% body mass in the harness at the end of the study.

Importantly, the intensity of the exercise was individualized such that the mice ran on the wheel at variable speeds according to their progression (4-10 rpm). The optimal goal for each set was to have the mouse "fail" at less than 60 seconds (~30 to no more than 75sec) by increasing the rpm. Because the training was individualized the time, distance, and weight were recorded for each set during every training session.

Failure

Failure was defined in 3 ways: *1)* the mouse was incapable of maintaining the rpm. The inability to maintain the velocity resulted in the vertical position of the mouse at the back of the wheel. *2)* The mouse stumbled 3 times. *3)* The mouse refused to run and grasped the grid of the wheel or threw off the harness (note, the mice participate voluntarily and can refuse to participate). The first time the vertical position of the mouse was observed, the mouse grasped the grid or the mouse stumbled (first and second time) the rpm was adjusted to a lower setting.

If the vertical position was observed a second time or the mouse stumbled a 3rd time, the set was considered complete (went to failure).

Noncompliance

A mouse was considered noncompliant when he refused to run or removed his harness. A mouse was given 3 opportunities to participate successfully in the specific training session. If not successful, the mouse was excused. As a result of this voluntary aspect of the training, if a mouse needed extra time to rest or to heal from a minor injury, it could refuse to train for a session without consequence to the study. If a mouse were to refuse to perform for 3 consecutive sessions, it would be removed from the study. No mice had to be removed from the study for repetitive noncompliance.

Outcome Measurements

Tissue and Cellular Response to Training

Muscle Function- Contractility

In vitro contractile physiology has been previously published (Graber, 2013). In brief: The soleus (SOL) and extensor digitorum longus (EDL) were isolated and perfused with 95% O_2/5% CO_2 in heated (25°C) Krebs solution. The muscle was tied to a force transducer (Aurora 300b) with 4-gauge silk suture attached at either myotendinous junction and suspended between two platinum electrodes. Using an Aurora High Power Bi-Phase Current Stimulator and a Dual-System

Signal Interface controlled by DMC (v.4.1.6), the muscles were stimulated in various protocols to examine peak twitch force (P_t), optimal length (L_0), pre-load tension, maximum isometric contractile force (P_0), and force/max force (P/ P_0) from 10 Hz to 180 Hz stimulus frequency. After P_0 was determined, the load clamp technique was used to determine velocity of contraction. The force transducer was set at various percentages of P_0 (10%-90%) and the muscle was set to L_0 and maximally stimulated at the frequency determined to produce P_0. Upon producing enough force to overcome the set load (clamped at 10-90% P_0), the muscle concentrically contracted against the load. Velocity of contraction was determined by finding the first derivative of the time-distance curve derived from the muscle contraction. Maximum unloaded velocity was calculated using a derivation of the Hill Equation [$(V+b)(P/P_0 \pm a/P_0) = b(1+a/P_0)$, a and b are constants, V = max. velocity at fractional load] (9). The data was curve fit (only $r^2>0.98$) using MatLab. Data presented here only reflects the determination of SOL contractile parameters.

Muscle Hypertrophy

Soleus Wet Mass

After *in vitro* physiology the soleus was blotted dry and massed. We determined physiological cross-sectional area (PCSA) with the average density of skeletal muscle by using the standard formula: PCSA (cm^2) = Muscle mass (g) / [L_0 (cm) * 1.06 (g/cm^3)].

Fiber Size-Histochemistry of Plantaris Muscle

These methods have been previously published (Graber, 2014). In brief: A subset of plantaris muscles was flash-frozen in isopentane, then liquid nitrogen. 10 micron sections were sliced as cross-sections on a cryostat and subsequently stained with hematoxylin and eosin (H&E). The sections were imaged with a microscope and then the perimeter of each fiber was circled using ImageJ to determine cross-sectional area.

Anabolic Signaling

Western Blotting technique was used to probe for the relative abundance in the level of phosphorylation in Akt (at the threonine 308 and the serine 473 sites) and in p70s6k (at the threonine 389 site). A brief description of the procedure follows.

Sample Preparation

The muscle (quadriceps femoris) was removed from the mouse while under deep anesthesia and instantly frozen in liquid nitrogen, then stored at -80° C. Homogenization was done by cutting an approximately 0.02 g piece of muscle, massing it, dicing it into 1 mm cubes and then putting it in a chilled glass homogenization tube. The ice cold Mueller buffer (consisting of ultra-purified H_2O, 0.05 M HEPES (pH 7.4), 0.1% V/V Triton-X 100, 0.004 M EGTA (pH 8.0), 0.01 M EDTA (pH 8.0), 0.015 M $Na_4P_2O_7$, 0.1M β-glycerophosphate (β-glycerophosphate disodium salt hydrate), 0.025 M NaF, 0.005 Na_3VO_4 (sodium

orthovanadate), 0.002 M PMSF (phenylmethanesulfonyl fluoride), 5 µg/µl leupeptin, 1 µg/µl pepstatin, 5 µg/µl aprotinin) was then added to the tube at a concentration of 100 µl of buffer / 100 µg of muscle tissue. The tissue was then homogenized, while kept in ice, using a low speed (300 RPM) for a total of 1 minute (forward at 70, 80 and 90% speed for 10 seconds each and then switch to reverse at 70, 80 and 90% speed for 10 seconds each). The supernatant was then carefully transferred to an Eppendorf tube, kept ice-cold at all times and then centrifuged 10 minutes at 10000 rpm at 4°C. The supernatant was then collected (avoiding any floating fat and without disturbing the pellet), measured and put into a second tube (retaining the pellet fraction as well). The chilled diluent buffer (consisting of 50% V/V glycerol, 0.05 M Na_4PO_7, 0.0025 M EGTA (pH 8.0), 0.001 M β-glycerophosphate, 0.002 M PMSF, 5 µg/µl leupeptin, 1 µg/µl pepstatin, 5 µg/µl aprotinin) was then added at a concentration of ½ the volume of supernatant obtained. The resulting mixture was then vortexed for 10 seconds on high, centrifuged for 5 seconds at 2000 RPM and stored at -80° C. Protein concentration was determined with the bicinchoninic acid method using the kit (product # 23225) from ThermoScientific, Rockford IL.

Western Blotting

For the Akt, electrophoresis was run on 10% acrylamide gels with a protein load of 22 µg on a minigel system (Hoefer SE250, Holliston MA) at 200 mV for approximately 1.5 hours using a water cooling system set at 12°C and standard

Laemli buffer. The same blot control sample was added in the last lane on every gel. Positive and negative controls were (#9273, Cell Signaling Technology, Danvers, MA) and the molecular weight ladder was Kaleidoscope (Biorad). The gel was then transferred to a PVDF membrane blot using a Biorad semidry transfer system (#170-3940), set at 14V for 24 minutes with Bjerrum and Schafer-Nielsen transfer buffer (pH 9.2 , ultrapure H_2O, 48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% V/V methanol). The blots were blocked in 5% dry milk/TBS-T for 1 hour at room temperature (RT), and washing steps (4x 5 minutes at RT) used TBS-T. The blots were incubated with the primary antibody dilution in sealed baggies on a rotator at 4°C overnight. The next day the blots were washed again and incubated for 1 hour at room temperature with the secondary antibody (goat anti-rabbit IgG, Cell Signaling Technology #7074s, 1:1000 dilution in 5% BSA/TBS-T with1% goat serum). The blots were washed again, the substrate was applied (Western Dura-Signal Plus, ThermoScientific) for 5 minutes, and were imaged on a Biorad Chemidoc XRS. The images were then analyzed for densitometry using QuantityOne software (BioRad).

Each blot was first probed with the phosphorylated antibody and then the same blot was stripped and re-probed with the Akt antibody. The blot was then re-stripped and re-probed for lane control with a GAPDH antibody. Antibodies and dilutions were: 1:500 p(THR308)-Akt, 1:1000 Akt, 1:5000 GAPDH (diluted in 5%

BSA/Tris Buffered Saline-0.1%Tween (TBS-T) (antibodies from Cell Signaling Technology: #2965, #9272, #14C10, respectively).

The p(THR389)-p70S6K and the p70S6K blots were run in a similar fashion. The electrophoresis was performed using a Biorad running at V for 45 minutes. The antibodies were diluted 1:500, p-p70S6K and 1:1000 p70S6K, (Cell Signaling Technology, #9205, #9202, respectively).

Whole Body Adaptations to Training

Training Effect- Whole Body Training Physiology

We tracked the individual performance of the mice over the training period. Training force, normalized training force, training power and normalized training power were all tracked through the duration of the study. See **Table 2** for definitions and calculation equations.

Animal Functional Performance

Animal performance measures are described in detail previously (Graber, 2013). Because the maximum ability of the mice is necessary to assess performance, we tested each mouse three times and then used the average of the two best scores as the outcome measurement (therefore excluding the worst time). In brief:

Rota-Rod tested overall motor function (balance, coordination, stamina, power) using the acceleration mode, from 4 RPM to 40 RPM over 5 minutes (Lsi Letica Rota-Rod R/S). The latency to fall from the device was recorded. Mice were acclimated to the device by doing random sessions 3x/day for 3 days prior to the testing day.

Grip Test is an indicator of muscle strength and stamina, determined by the inverted cling grip test (custom testing device). The animal was placed on a wire grid lid and the lid was then closed to invert the mouse. The latency to fall from the lid (to the padded floor of the device 20 cm below) was recorded as the outcome measure.

Statistics

Data presented as means ± standard error, as appropriate. Repeated measures general linear model (compare within subject in the rotarod and grip test), Student's t-tests (independent and paired), ANCOVA and linear regressions use $\alpha<0.05$ as significant and $\alpha<0.10$ as showing strong evidence to refute the null hypothesis and representing a trend. Least Significant Difference (t-test) used for post-hoc testing (only 2 groups). ANCOVA adjusted for body mass in grip test and rotarod. Pearson correlation used to determine relationship between variables. 2-sample Kolmogorov-Smirnov and the independent samples median test were used to compare distributions of plantaris muscle fiber cross-sectional

area. Symbols: “*” denotes $p<0.05$, significant and “$^{\#}$” denotes $p<0.10$, trend. IBM SPSS v20 used for statistical analysis.

RESULTS

Tissue and Cellular Response to Training

Muscle Function- Soleus Contractility

Soleus (SOL) Maximum Isometric Force (mN, P_0) and Normalized Force (P_0/gbm)

Although absolute P_0 and P_0 normalized to the cross sectional area of the muscle (P_0/PCSA) were not different, the normalized force production in the exercise group (8.35 ± 0.34 mN/gbm) was 11% greater than the control group (7.55 ± 0.32 mN/gbm) (T-test, p=0.097, **Figure 3A**).

SOL Contractile Velocity

The velocity of contraction in the exercise group was faster when measured while contracting against the higher percentages (higher loads) of P_0 (60%P_0, +17%, p=0.044; 80%P_0, +36%, p=0.024; 90%P_0, +44%, p=0.051). An indicator of the shape of the force-velocity curve based on the Hill equation, a/P_0, was 21% lower in the exercise group than in the control group (0.018±0.002 and 0.023±0.002 respectively, p=0.055)(**Figure 3B**).

SOL Power Production

The shape of the force-power curve shifted to the right and up with exercise. With exercise, power production increased 39% at 80%P_0 and 47% at 90%P_0 (p=0.019 and 0.048, respectively, Figure 5C), representing the upward shift. The

$\%P_0$ where peak power occurred increased by 7% with exercise (control 27.3±0.6% and exercise 29.2±0.5%, p=0.045), representing the rightward shift. Although the $\%P_0$ where peak power occurred increased, P_{max} was not statistically increased with exercise (exercise, 97.7±10.5 mN*fl/s, and control, 89.1±4.7 mN*fl/s, p=0.47). (**Figure 3C**)

Muscle Hypertrophy

SOL Wet Mass (Figure 4A)

The mean SOL muscle mass of the exercise group (14.5 ± 0.8 mg) was 15% larger (p=0.019) than the control group (12.6 ± 0.7 mg). When the SOL was normalized to the size (end point body mass) of the animal, the exercise group (0.39 mg/gbm) was 26% larger (p=0.004) than the control group (0.31 g/gbm).

Fiber cross-sectional area of the Plantaris (Figure 4B)

Single fiber cross-sectional area increased 4% with exercise (exercise: 2006.4 ± 16.7 μm^2, n=3175; control: 1933.7 ± 22.8 μm^2, n=1762, T-test, p<0.001). A close analysis of the distribution of the cross-sectional areas revealed a rightward shift (2-sample Kolmogorov-Smirnov, p=0.001) with exercise, notably more large cells were present in the exercise group. Specifically, the median cell cross-sectional area in the exercise group (1920.5 μm^2) was 7% larger than the control group (1793.0 μm^2) (independent samples median test, p=0.002).

<u>Anabolic Signaling</u>: Akt and p70S6k

The exercise group demonstrated an increase of 30% in phosphorylation of p70s6k (p-p70s6k) at the Threonine 389 position (p=0.059) when compared to the control group (**Figure 5**). The exercise group demonstrated no significant increase in phosphorylation of Akt at the Threonine 308 position (p=0.155) when compared to the control group (**Figure 6**). There was no significant change in the ratio of either p-p70s6k/p70s6k or p-Akt/Akt (data not shown).

<u>Whole Body Adaptations to Training</u>

Training Force (mN), Normalized Training Force (mN/gbm), Training Power (mW), and Normalized Training Power (mW/gbm):

Training Force improved over the training sessions (Force = 4.56 * (training session) + 380.5, R=0.992, r^2=0.984, p<0.001) (**Figure 7A**). Normalized Training Force also improved (Force = 0.126 * (training session) + 0.703, R=0.992, r^2=0.984, p<0.001) (**Figure 7B**). Training Power (Power = 0.503 * (training session) + 34.9, R=0.936, r^2=0.879, p=0.002) and Normalized Training Power (Normalized Power = 0.014 * (training session) + 0.963, R=0.937, r^2=0.879, p=0.002) both increased over the training period. (**Figures 7C, 7D)**.

Mean Training force increased by 39.3% between session 3 (387.1 ± 9.6 mN) and session 31 (539.3 ± 9.6 mN), an increase of 152.2 ± 0.0002 mN (p<0.001). Likewise, mean normalized training force increased by 443% between session 3

(0.836 ± 0.021 mN/GBM) and session 31 (4.54 ± 0.12 mN/GBM), a difference of 3.70 ± 0.094 mN/gbm ($p<0.001$). Mean training power increased by 30.5% between session 3 (38.0 ± 1.8 mW) and session 31 (49.6 ± 1.3 mW), a difference of 11.5 ± 1.7 mW ($p<0.001$). Mean normalized training power increased by 23.4% from session 3 (1.05 ± 0.04 mW/gbm) to session 31 (1.37 ± 0.03 mW/gbm), a difference of 0.322 ± 0.048 mW/gbm ($p<0.001$).

Animal Functional Performance

For the functional performance measures, 2x2 Repeated Measures ANCOVA (2 groups, 2 times, pre- and post- intervention, time in seconds) and 1-way ANCOVA (mean % change), adjusted for body mass, were used for comparisons.

Rotarod (overall neuromuscular performance, balance, coordination, stamina, power)

The exercise mice increased function (%change) compared to the control group ($p=0.031$), adjusted for mass (**Figure 8A**). Notable, the control mice had reduced function in number of seconds prior to falling (-20%, $p=0.037$) whereas the exercise mice did not change significantly (+12%, $p=0.573$) (**Figure 8B**, repeated measures, adjusted for mass).

Inverted Cling Grip Test (overall muscle strength and stamina):

The control mice lost 52% of grip function, but the exercise mice only lost 19% function. This represents a 212% reduction in ability in the control mice compared to the exercised mice. The changes, however, were not significantly different after adjusting for body mass (**Figure 8C**).

The control mice lost functional ability, but ability was relatively preserved in the exercise group. In terms of seconds, the control mice (n=9) lost a mean of 143.8 seconds (within group, p=0.006) compared to a 16.6 loss (within group, p=0.372) in the trained mice (n=6) (between groups, p=0.046) (repeated measures, **Figure 8D**).

The percentage difference between the grip test before and after the intervention period was correlated with both the mass of the mouse pre-intervention (from simple linear regression, $p<0.001$) and with the mass after intervention ($p=0.023$). Body mass was not correlated with grip test changes in seconds within subject (repeated measures).

Animal Mass

There was no change with the intervention in the relative proportion of mass between the control and exercise group. Specifically, at baseline, body mass was

significantly different between the two groups with the control mice being 8.4% heavier (p=0.037). The mean mass of the control after the intervention was 8.6% greater than the mass of the trained group, p=0.001. Within each group, there was a 25% change between the pre- and post-intervention (control p=0.001, trained p=0.003).

There was a significant association of SOL muscle mass to body mass in both the trained group (R=0.796, p=0.029) and the control group (R=0.575, p=0.032).

DISCUSSION

Current hypertrophy models used to investigate cellular mechanisms in mice are less representative of voluntary human weight (resistance) training. Thus, the main purpose of this study was to produce and validate a voluntary mouse exercise protocol that mimics human weight training as would be performed in the gymnasium. Weight training improves performance and physiological parameters including: muscle strength, endurance, power output, speed, balance, coordination, motor performance, and induces hypertrophy (Kraemer, 2005; Kraemer, 2000). Thus, we hypothesized that many of these same adaptations would occur in our resistance trained mice, and, if so, would successfully validate the protocol as a mimic of human resistance exercise.

This study had two main findings. *1)* Positive results occurred after undergoing the resistance training protocol in most of the traditional outcome measurements of muscle hypertrophy, muscle force, velocity and power, increased anabolic signaling in the p70S6K pathway, gross neuromotor function (rotarod), and in the derived measurements of training physiology--training force and power, both absolute and normalized to body mass. *2)* These results demonstrated the protocol is a mimic of human weight lifting, with both face and construct validity.

Training Specificity

Training specificity refers to the actions of an exercise producing adaptations that facilitate functional improvement in activities similar to the exercise (Morrissey, 1995). For example, in order to become better adapted for sprinting, practicing by running at a rapid pace induces more positive effects than would long-distance swimming. Specificity can also refer to the muscle groups targeted by an exercise. For example biceps curls activate, stimulate and induce plasticity in the biceps (arm flexor), but would do little or nothing to the gastrocnemius (plantar flexor). Hence, in the current study training specificity refers to the mode of exercise (running with a weighted pack), which muscles are influenced, and what functional changes would be expected to occur. Specifically, the outcome measures more closely related to the exercise modality were expected to have greater relative change after training (rotarod would improve more than the inverted cling grip test). The muscle groups used for plantar flexion (gastrocnemius complex consisting of the gastrocnemius, soleus and plantaris) or leg extension at the knee (quadriceps) would be the most affected muscles. Thus, within the limits of training specificity we expected to see evidence of improvement in the outcome measures we selected as an indication that our training protocol for mice was indeed a mimic of human weight training.

Tissue and Cellular Response to Training

SOL *in vitro* Contractile Physiology (absolute strength and power production)

The SOL improved in force output, velocity of contraction and power output, which is expected because as a plantar flexor, the SOL was directly activated during the exercise. These improvements have, of course, been reported in humans after resistance training (Pillard, 2011; Kraemer, 2000).

The direct *in vitro* stimulation of the muscle is similar to a 1 rep maximum test in a human study because it is measuring the absolute strength of the muscle. Notably, the reported increases in velocity and power output occurred at the upper limits of the force-velocity and the force-power curves. These increases in ability to perform more difficult tasks (e.g. at higher percentages of maximum force) would be considered a hallmark of weight training programs, where one of the most important goals is to facilitate performance under increasingly heavy load.

Hypertrophy (whole muscle and cellular):

Muscle Size (SOL Mass) and Fiber Size (Plantaris)

The exercise protocol resulted in both an increase in SOL muscle mass and single fiber hypertrophy in the Plantaris. This result was expected due to training specificity. Both muscles are plantar flexors and are activated by the exercise

modality. The muscle mass outcome measure represents an increase in all tissues within the muscle (muscle fibers, nerves, fat, connective tissue), whereas single fiber hypertrophy (the increase in muscle fiber cross-sectional area) reflects increases of only the sarcoplasm and contractile elements within the fibers. The increase in both muscle and fiber size is consistent with reported human studies of resistance exercise programs (Mitchell, 2013; McCall, 1996; Tesch, 1988).

The fiber type composition of the muscle influenced the extent of hypertrophy. Type II fibers respond robustly to resistance training, whereas type I fibers typically hypertrophy to a much smaller extent (Tesch, 1988). In mice, the SOL is composed of both fiber types, approximately 60% type I (Burkholder, 1994). In spite of this large amount of type 1 fibers, there was a significant mass increase in the SOL with training in our current study. In contrast, while the soleus muscle in the rat also has both fiber types, the fiber type percentages are widely different (approximately 90% type I) (Armstrong, 1984). Hence, the literature on rat resistance training models report limited overall response in the SOL, presumably because of the larger percentage of relatively hypertrophy resistant type I fibers. In one example, weights were attached to a tail cuff and the rats climbed a ladder. This study failed to produce SOL hypertrophy at a significant level. However, when analyzed separately, the SOL type 2 fibers showed some

evidence (trend, p=0.09) of increased cross-sectional area after training (Deschenes, 2000).

Anabolic Signaling Pathways

In order to have muscle hypertrophy there must be an increase in anabolic signaling that initiates RNA translation and protein synthesis. The mTORc1 pathway is central to regulating protein synthesis (Glass, 2010) and is responsible for phosphorylating p70S6K, which must be phosphorylated at the Threonine389 position for translation to occur. Measuring signaling pathways is a way to examine whether anabolic signaling was increased by exercise at the time point in which the sample was gathered (one to two days post-training).

Indeed, the evidence of increased anabolic signaling (more overall phosphorylation of p70S6K) in the exercise group is consistent with basic biology--mTOR is stimulated by exercise to phosphorylate p70S6K, which downstream turns on protein translation (Hornberger, 2011; Baar, 1999). Training has been shown to result in increased p70S6K phosphorylation for up to 24 (in trained individuals) to 72 hours (in untrained people) after training (Drummond, 2010). A resistance training model of the squat for rats found increases in p70S6K phosphorylation after an acute bout of training that was significant at 12 hours, but not significant at 6 or 24 hours after the training session (Drummond, 2010).

We found no change in the phosphorylation of Akt at the threonine 308 site, at least at the time point tested. A study of anabolic response to acute bouts of exercise in humans showed an increase in phosphorylated AKT (308) that occurred one hour post exercise (Dreyer, 2010). It is therefore quite possible that our samples were taken during a time point that simply missed this particular signaling cascade. Traditionally, activation of Akt at this site is thought to occur as a result of upstream activation of PI3K by growth factors (such as IGF-1) (Glass, 2010). Recent research has suggested that mechanical loading does not directly activate Akt via PI3k to phosphorylate mTOR to initiate transcription, but rather acts (via an as-of-yet unknown action) by upregulating diaglycerol kinase (DGKζ) to facilitate binding of phosphatidic acid (PA) to the mTORc1 complex (You, 2014; Zanchi, 2008). Mechanical stimuli circumvent Akt activation of mTor. Thus, not finding change in Akt activation, after resistance training, is not inconsistent with a training-induced anabolic state. Future investigations should investigate the DGKζ and PA connection to mechanically induced increases in protein synthesis, as well as to take samples closely following the exercise bout (within 3 hours), to establish if the timeframe of phosphorylation at the Akt threonine 308 site was simply missed. Additionally, since Akt is also phosphorylated at the serine 473 site (by mTORc2), this additional signaling event should be examined.

Thus, we conclude the basic signaling pathways investigated give evidence that the stimulus of the training protocol resulted in expected signaling outcomes (increased phosphorylation of p70S6K at the threonine 389 site, which is required for downstream translation initiation). More research into resistance training-induced signaling pathways is needed. We suggest, beyond investigating DGK and other anabolic components, the catabolic side of the protein synthesis equation be addressed by examining E3 ligases such as MURF1 to determine if there is a corresponding decrease in catabolic signals to complement the increase in anabolic signaling.

Whole Body Adaptations to Training and Functional Performance

Training Physiology (training induced changes in force and power)

In order to assess whether the mice adapted positively in their ability to perform the exercise we recorded the weight used, distance moved, time and velocity of each mouse for every session. With this information we then calculated the force and power produced by the mice. Over time the mice were expected to gain increased ability, as their physiology adapted to the increasingly demanding exercise protocol. Indeed, we found that both training force and power increased over the sessions. These increases validated the ability of the training to stimulate responses in strength and power as would be expected after undertaking a weight training regimen.

Functional Performance

Rotarod (overall neuromuscular motor function)

We found that exercise increased rotarod ability. The rotarod tests multiple aspects of neuromotor and muscular function, and could be considered the equivalent of a human functional test. One such common functional test is the timed up and go (subject gets up from a chair and then moves a certain distance, with the outcome measure being the time elapsed) (Frenken, 2014), which would be expected to improve after an exercise regimen.

Running on the powered running wheel with a weighted harness is similar to running on the rotarod (training specificity), and utilizes many of the same muscles. We therefore expected a positive adaptation from the training. Improvement of this outcome measure in the exercise group contributed to validating the ability of the training protocol to invoke expected positive change in balance, coordination, endurance, gait speed, and power production. Resistance training in humans also shows improvement in these parameters (Kraemer, 2000).

Inverted Cling Grip Test (strength/endurance)

This outcome parameter is similar to a human pull-up test in scope (Graber, 2013). Based upon training specificity, we would not expect a large change in this parameter because the exercise, running with weights, is not similar to the

functional task, suspending upside down, and primarily stresses different muscle groups. The grip strength muscles were only partially activated during the motion of the exercise. In human terms, the situation is similar to training a group with push-ups and then testing them on pull-ups.

There was also high individual variability in this measurement (coefficient of variation in the training group was 463). In comparison to the exercise group, the control mice had a higher mean level of ability at baseline, driven by a few very high performers. The control mice lost much more ability than the exercise mice. The exercise mice had a relative preservation of function. Therefore, this measurement demonstrated that there was a reduction in functional decline with training. Because a lower amount of positive change may have been experienced by the exercise mice because of the lack of training specificity of the exercise for the functional task, a similar result could be expected in a similarly conducted human study.

In future studies, we suggest evaluating an additional grip test measurement. In this test the mouse is assessed for strength by having it grasp a trapeze-type bar attached to a force transducer and pulling it by its tail until it releases the bar (Aartsma, 2014; Smith, 1995). The maximum force when the mouse releases the bar is the outcome. It is possible that we may see less variation in the results

than in the inverted cling grip test because this measurement is less dependent upon body mass, although it does have a degree of user induced variability.

Caveats to Consider

In future work we propose the use of DEXA (dual-energy x-ray absorptiometry) to track body composition and bone mineral density as an additional outcome measure. We hypothesize that adjusting for lean body mass instead of total body mass will result in better estimation of muscle quality. In addition, the mice were not matched for body mass prior to initiating training. They were completely randomly selected to each group. In the future, prior to randomization and functional testing, matching by mass would be an improvement in study design.

CONCLUSION

We found that our resistance training protocol demonstrated evidence of improving many performance and physiological parameters including muscle strength, endurance, power output, speed, balance, coordination, motor performance, reduced loss of function in the grip test, and induced hypertrophy. The voluntary resistance training protocol for mice results in many of the same adaptations post-interventions that would be expected in a group of humans undergoing a training program designed with similar exercise principles (Kraemer, 2000; Kraemer, 2005). Thus, our strategy of using human principles of weight training to design our mouse protocol resulted in numerous expected

adaptations and supports that our objective of creating a mouse model mimetic of human weight training was successful. This validated protocol will serve a valuable future function in pre-clinical investigations and in basic science research surrounding resistance exercise adaptation. Our future direction will be to use this protocol on an aging mouse cohort to investigate its efficacy in the aged population and to discern if there is evidence of anabolic resistance to the stimulus in the elderly mice.

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Table Captions

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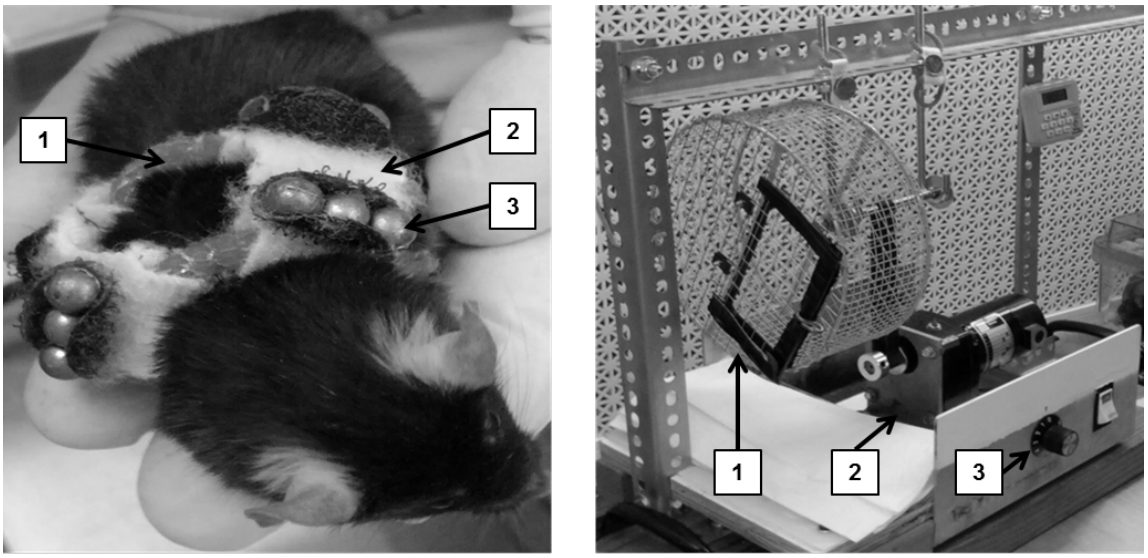
Table 1 Training Principles used in Protocol

Term	Explanation/Description	Application in Study
Progressive Resistance	Exercise performed with increasingly heavier weights over time	Increased weights every 3-5 sessions
Progressive Intensity	Exercise performed at a faster pace or with reduced resting time	~4-10 RPM, by individual ability
Rest Period	Time between sets to recover for the next set, typical strength emphasis rest is 4-5 minutes, less for hypertrophy	4-5 minutes rest
Frequency	Number of exercise sessions performed each week	1 session per day, 3 days per week
Sets	1 continuous bout of numerous repetitions of exercise	4-5 sets per session
Set Duration	Number of repetitions per set, Humans: hypertrophy 8-15 repetitions, power 3-6 reps, usually not more than 1 minute (15 reps at ~4 seconds)	~30 to <60 seconds, anaerobic, to equal typical human set duration
Training Period	Length of training. Many human studies are 3-4 months. First 1-4 weeks, mainly neural adaptions. Hypertrophy main effect after 4 weeks.	12 weeks
Warm-up Sets	Initial sets performed at very light weight and/or intensity to prevent injury and prime for heavier lifting	1 or 2 warm-up sets per session
Failure	The last repetition of an exercise that can be safely performed in good form	Stumble 3x or reach vertical position twice

Table 2 Training Physiology Definitions Units: mN=milliNewtons, g=acceleration due to gravity 9.8 meters/second, gbm=grams of body mass, mW=milliWatts, mJ=milliJoules

Training Parameter	Definition	Equation
Training Force (mN)	Total mass of the mouse plus weight used in each session.	*Mass in grams (mouse mass + weight and harness) * acceleration (g)*
Normalized Training Force (mN/gbm)	Total weight the mice lifted (including body mass) per gram of body mass.	*[mass in grams (weight harness + mouse) * acceleration (g)] / grams of body mass (gbm)*
Training Power (mW)	Work performed per second.	*Training Power (mW) = Work (mJ) / time (s)* *[Work (mJ) = Training force (mN) * distance run (m)];*
Normalized Training Power (mW/gbm)	Power produced per gram of body mass.	*[work (mJ) / time (s)] / gbm*



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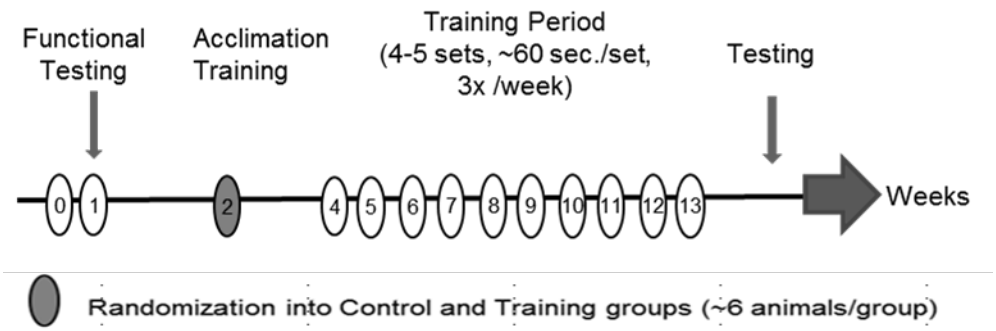

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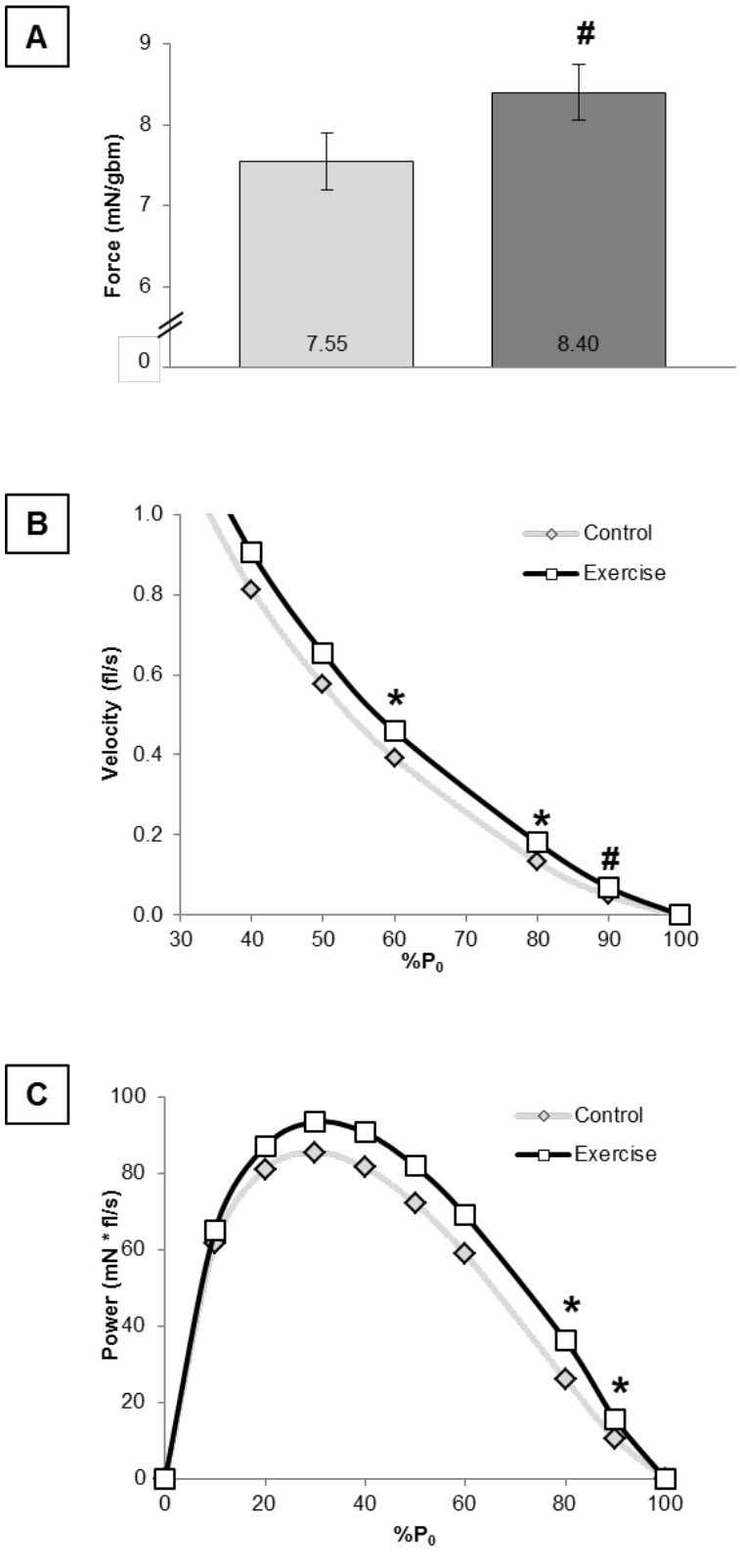

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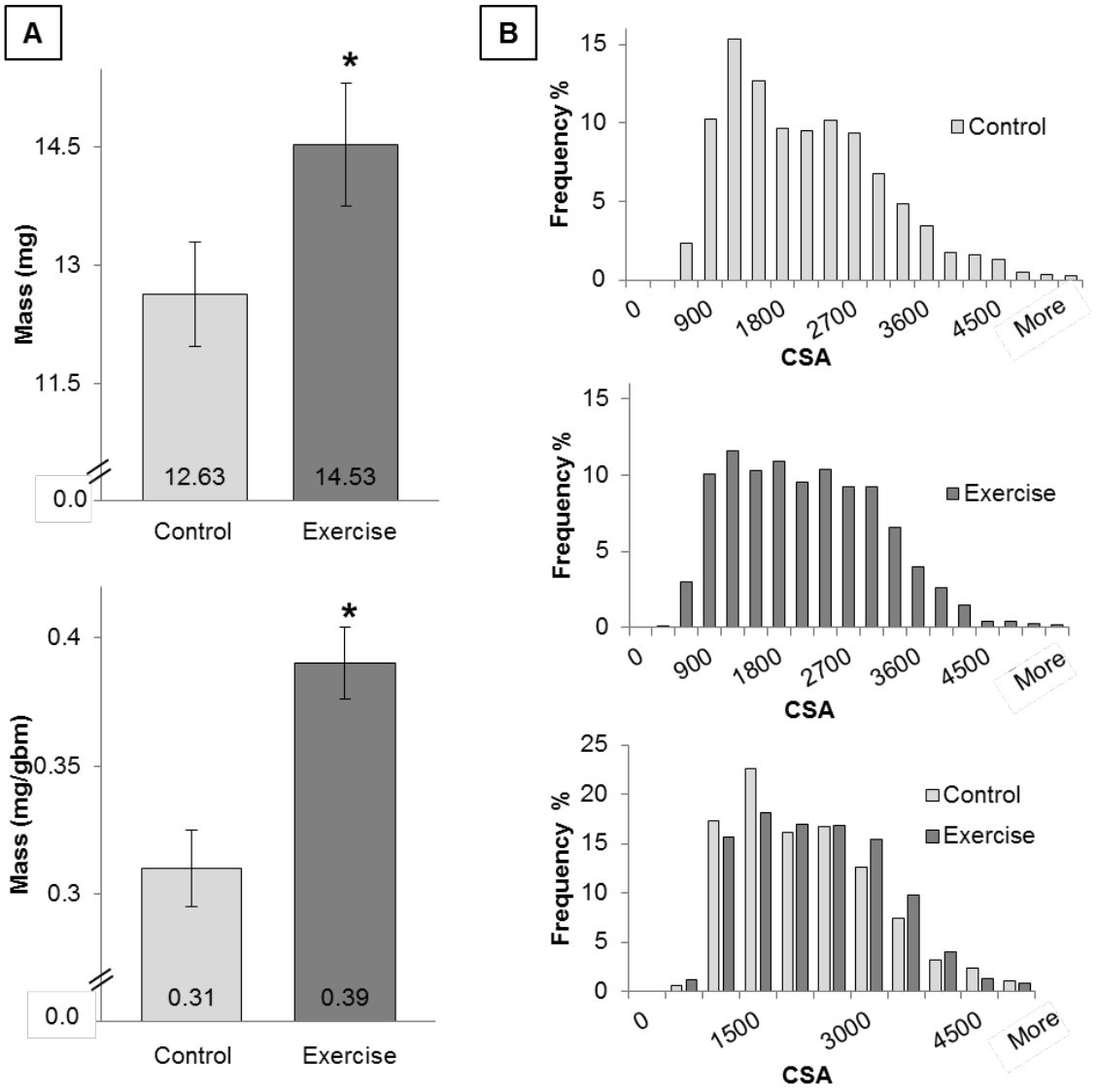

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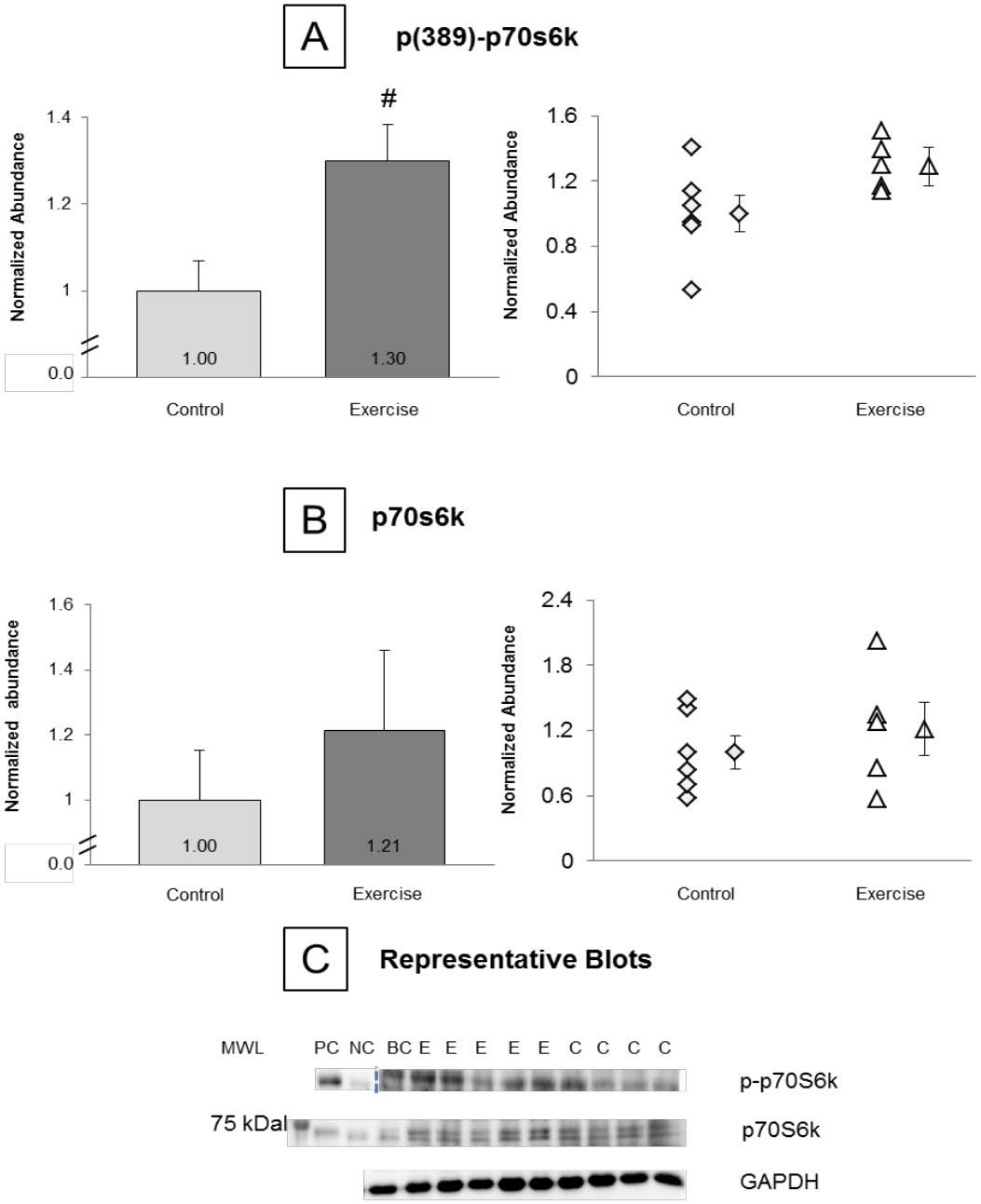

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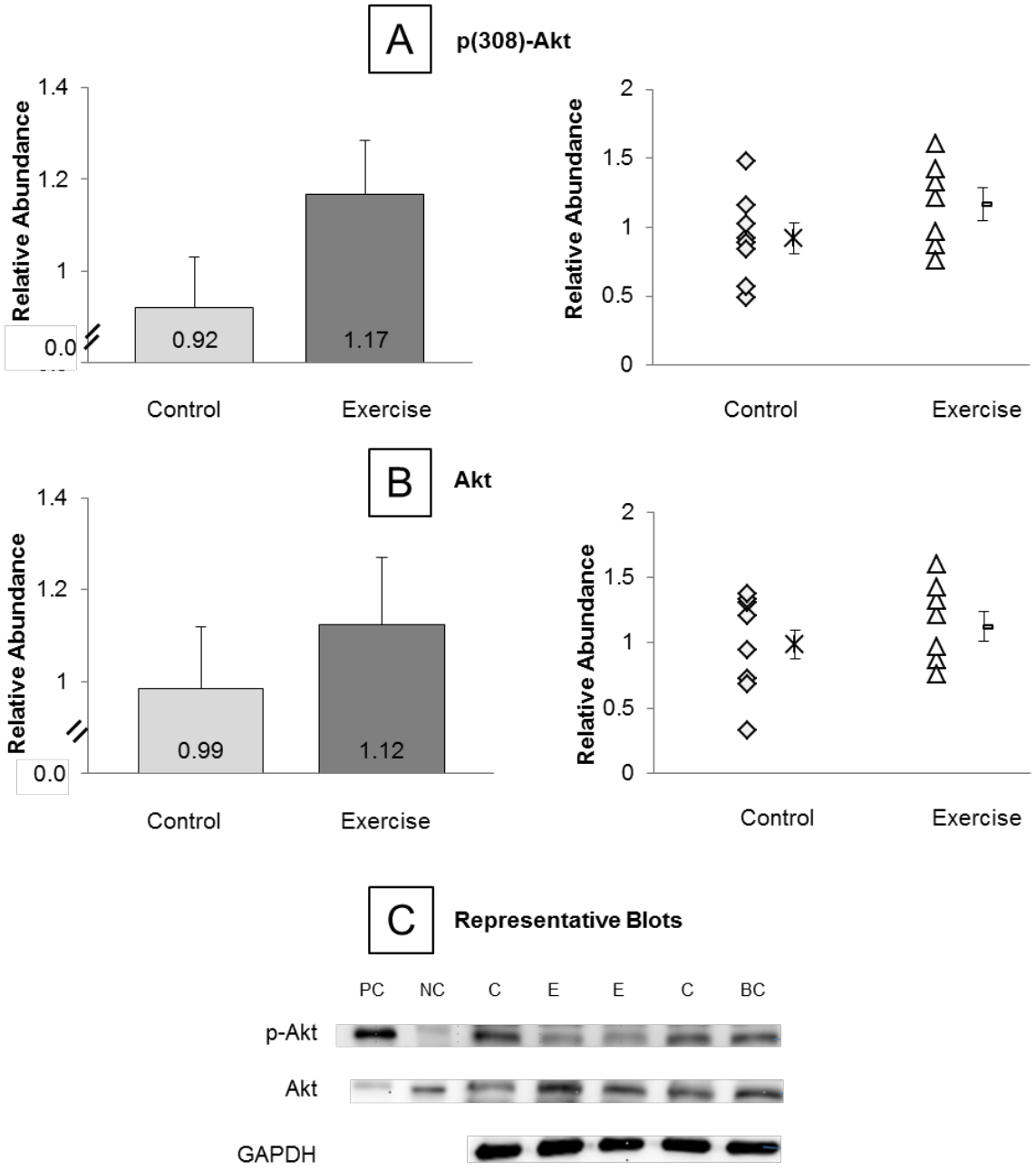

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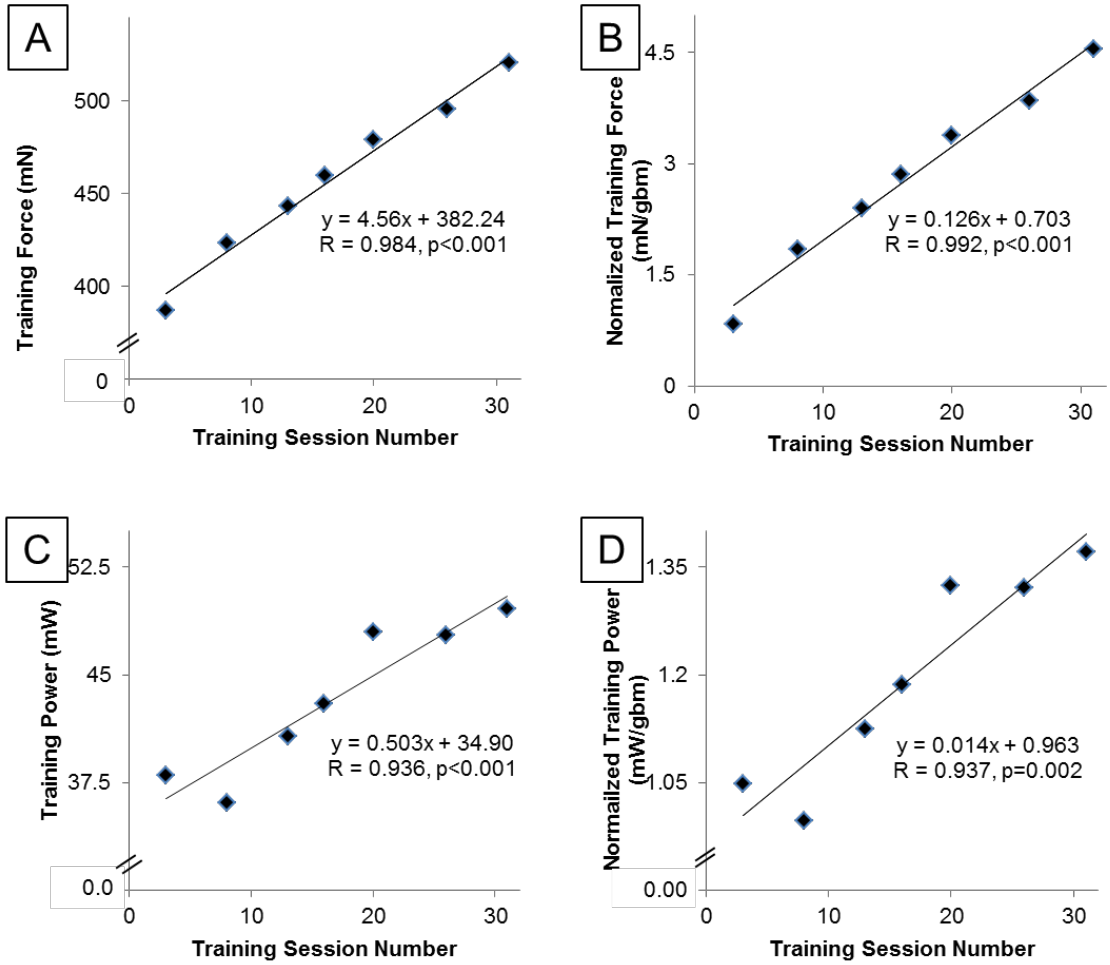

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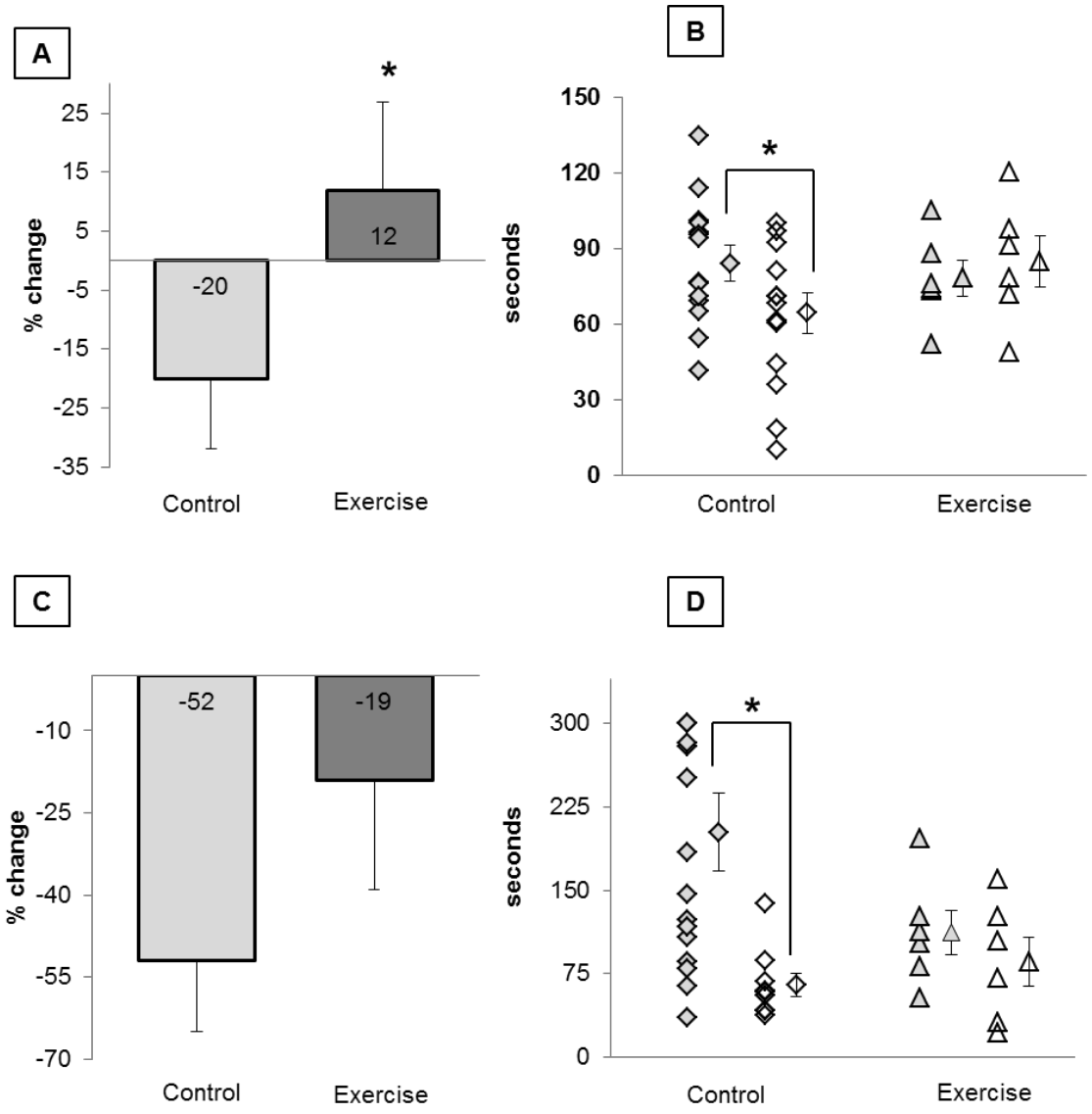

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Interlude 3

Chapter 4 described the voluntary resistance training protocol for mice. Because our ultimate purpose is to establish an exercise model to investigate sarcopenia and treatments for sarcopenia, the next logical step was to apply the newly validated protocol to a cohort of older mice. These mice were 28 months of age at the conclusion of the study--similar in age, in terms of lifespan, to 78-80 year old humans.

In Chapter 5, *Voluntary Resistance Training in Elderly Mice and Signs of Anabolic Resistance*, we tested the hypothesis that elderly mice undergoing this training would have positive adaptations, but the effects would be somewhat modulated, or muted. In other words, we expected some evidence of anabolic resistance.

If the older mice had fewer, or of lesser magnitude, positive adaptations than the adult mice we concluded this was an age-associated blunting of response to anabolic stimuli. Using the same outcomes measures as in Chapter 4, with the addition of DEXA to determine body composition, we determined that indeed less adaptation was achieved in the older mice, thus providing evidence to support our hypothesis.

Chapter 5

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Voluntary Resistance Training in Elderly Mice and Signs of Anabolic Resistance

Ted G Graber, LaDora V Thompson
Department of Physical Medicine and Rehabilitation
University of Minnesota Medical School

TGG primary author of manuscript and study designer, conducted all animal training, performed assays, collected and analyzed all primary data

LVT guarantor of study, assisted in study design and data interpretation, major contribution to manuscript writing, scientific manuscript review, funding

Running Head: Resistance Training with Elderly Mice

Corresponding Author: LaDora V. Thompson
Email: thomp067@umn.edu, Phone: 612-626-5271 (office), 612-624-2436 (fax)
University of Minnesota Medical School Department of Physical Medicine and Rehabilitation
Program in Physical Therapy
Rm 366A ChRC
426 Church Street SE
Minneapolis, MN 55455

Abstract

Sarcopenia, age-related loss of muscle mass and strength, contributes to frailty onset, loss of independence and reduced quality of life in the elderly. Resistance training is an accepted treatment that slows the progression of sarcopenia. We previously validated mouse exercise protocol that mimics human weight training. In this study we hypothesized that applying the protocol to a cohort of aged mice would demonstrate evidence of anabolic resistance when the outcome measurements were compared to those of the adult mice from the previous study.

An age cohort of male C57BL/6 mice was used (28 months of age at study conclusion; n= 24). The mice were randomly separated into control and trained groups. The trained groups used progressive resistance (via a weight harness) and intensity (~4-10 RPM) on a custom motorized running wheel (possible speed from 1-10 RPM). The mice trained on a program similar to a human beginning workout regimen (4-5 sets/session, 3 sessions/week, for 14 weeks).

There was evidence of anabolic resistance because the elderly mice examined in this study did not improve to the same degree as the adult group from the prior investigation. Overall, we did find evidence of significant positive change after undergoing the training intervention in many outcome measurements, including: overall neuromuscular function (rotarod), strength/endurance (inverted-cling grip test), training physiology (force output per session), muscle size (soleus mass), anabolic signaling (p79s6k phosphorylation), and power production (*in vitro* contractile physiology). Future research is needed to develop strategies to improve exercise response in elderly subjects.

Keywords: Sarcopenia, Resistance Training, Mice, Anabolic Resistance, Exercise Physiology, Muscle

INTRODUCTION

The world is experiencing an unprecedented demographic shift with the number of people over the age of 60 having quadrupled since 1950 and estimated to triple from again by 2050 (UNDESA, 2011). With increasing age come the onset of age-associated disease, syndromes and potential disability. Sarcopenia, the age associated loss of muscle mass and strength, contributes to the development of frailty and directly leads to a reduced quality of life, loss of independent living and increased mortality. Sarcopenia at the clinical level of impairment is estimated to have a prevalence of approximately 5-13% in 60-70 year olds, but rises to up to 50 percent of seniors over 80; though estimates vary widely across the literature (Janssen, 2002; Cruz-Jentoft, 2014). With no current cure for sarcopenia, it is imperative to investigate potential treatment strategies to both extend healthspan and independent living, and before our healthcare and assisted living infrastructure is crippled with masses of sarcopenic and frail elderly.

Resistance training has been shown to improve muscle mass, strength, and function in even the oldest and most frail individuals (Fiaterone, 1990; Fragala, 2014). Anabolic stimuli, such as exercise or nutrient intake, have a reduced effect in older individuals, contributing to sarcopenia (Haran, 2012). Targeting the multi-level etiology of sarcopenia and anabolic resistance by combining nutritional

and/or pharmaceutical interventions with exercise training may be a good strategy to improve exercise response in older individuals.

In order to research the underlying mechanisms of anabolic resistance and to begin to develop/test new strategies to combat sarcopenia and frailty we need to develop animal models. In the current study, we apply our voluntary resistance training protocol for mice (Graber, 2015 B) to a cohort of elderly mice (50% survival, 28 months old at study completion; which is roughly equivalent to 78-80 year old humans) (Graber, 2013) and assess a carefully selected battery of outcome measures designed to determine the efficacy of the training protocol to induce positive changes in the old mice. Many of these measurements were also incorporated into our previous work with adult mice and so we are able to directly compare the data from the elderly to the adult data from the earlier investigation. We hypothesized that applying our protocol to a cohort of aged mice would demonstrate evidence of anabolic resistance in comparison to the adult mice from the previous study.

While the elderly mice do, in fact, improve in many of the outcome measurements, they do so to a lesser extent than the adult animals did. They also show very little improvement or no change in some of the measurements. We present evidence herein that the mice may be experiencing anabolic

resistance, in that they are receiving a lesser response than the adult mice to the same exercise stimuli.

METHODOLOGY

Animal Model

The mice used were C57BL/6 males purchased from the NIA Aging Colony (elderly, 28 months old at study completion=75% survival). The mice were randomly selected to exercise (n=9) or control groups (n=12). For purposes of comparison with the elderly mice, adult mice described in (Graber, 2015 B) are also reported (n=15), a detailing of direct comparisons with 2x2 ANOVA is in the Supplement, and a comparison of the significant effects of exercise in the adult and old mice is detailed on Table 3. Both comparisons are discussed briefly. All mice were handled and treated humanely in accordance with approved IACUC protocols. All of the mice were fed *ad libitum and* group housed at 22°C with a 12-hour light, 12-hour dark cycle. The mice were weighed both prior to and after completion of the training period. BMI (body mass index) was calculated as mass (kg) / length^2 (m^2).

Training Equipment and Protocol

This study uses the resistance training protocol that was previously validated (Graber, 2015 B). The specialized training equipment used was also previously described. Briefly:

The goal of this study was assess the effects of the resistance training protocol as previously described in adult mice on a cohort of elderly mice. The protocol

itself was designed a mimetic of human weight training and emulates the human bodybuilding exercises of “walking lunges” or the “farmers walk”. It could also be compared to a “weighted sprint” at lighter loads. The custom equipment was used--a weight harness and a powered running wheel.

The weight harness consisted of two padded elastic bands connected with two Velcro strips. Lead shot (weights of varying mass) could be loaded onto the Velcro strips to increase the load carried by the mouse. The powered running wheel was made from a caged-in 25 cm running wheel (with hinged door) attached to an electric motor, with a speed controller from 1-10 RPM to increase intensity. Two critical pieces of equipment were custom-designed and fabricated. The mice underwent an adaptation period to the harness and running wheel and then resistance trained for 14 weeks (**Figure 1**).

The resistance training program was designed with principles of human weight training in mind (Garber, 2011; Desalles, 2009; ACSM, 2009; Bird, 2005; Kraemer, 2004; Schoenfeld, 2010): progressive resistance/intensity (ACSM, 2009; Kraemer, 2004), number of sets per day (ACSM, 2009; Krieger, 2009), set duration (Toigo, 2006), warm-up sets (ACSM, 2009), training frequency (ACSM, 2009), 2009), and rest between sets (ACSM, 2009; Desalles, 2009). Specific of the training regimen can be found in Chapter 4.

It is important to note that just as human subjects in an exercise study would progress at different rates and start at a different ability level, so did the mice. Because of this it was necessary to progress each mouse individually which most often meant exercising individuals at the same resistance but using different intensity (speed, modulated by controlling the RPM of the running wheel) and/or set length. However, in some instances varying of weights were necessary as some mice progressed either faster or slower than the norm. The mass of the weight carried, time elapsed, the distance moved and the speed was recorded for every second of every session of exercise for each mouse throughout the study. The mice ran to failure during working sets (defined as non-warm-up sets) with failure being defined in three ways: 1) the mouse stumbles three times (RPM reduced after the first 2 instances, 3rd occurrence equals failure), 2) the mouse refuses to run further and grasps the wheel (1st time RPM reduced, 2nd time equals failure), or 3) the mouse cannot maintain velocity and reaches a vertical position at the back of the wheel (1st time the RPM is reduced and the 2nd time the mouse is finished for that set). Since this is a voluntary protocol, the mice can refuse to participate. They did so mainly in two ways: 1) grasping the wheel and refusing to run (see failure description above), or 2) “shrugging” out of the harness. Mice were allowed 3 opportunities to participate in the training session. After the 3rd refusal, the mouse was recorded as noncompliant on the training record and was returned to his cage. Any mouse that refused 3 consecutive

training sessions was to be removed from the study. In the cohort of the current study no mice had to be removed for continued noncompliance.

O**UTCOME MEASUREMENTS**

The same outcome measurements were used as previously reported (Graber, 2015 B) with some additions. A brief description of each measurement follows.

Whole Body Adaptations to Training

Animal Functional Performance

Animal performance measures are described in detail previously (Graber, 2013). Because the maximum ability of the mice is necessary to assess performance, we tested each mouse three times and then used the average of the two best scores as the outcome measurement (therefore excluding the worst time). In brief:

Rota-Rod tested overall motor function (balance, coordination, stamina, power) using the acceleration mode from 4 RPM to 40 RPM over 5 minutes (Lsi Letica Rota-Rod R/S, **Figure 2 A**). The latency to fall from the device was recorded. Mice were acclimated to the device by doing random sessions 3x/day for 3 days prior to the testing day.

Grip Test is an indicator of muscle strength and stamina, determined by the inverted cling grip test (using a custom testing device, **Figure 2 B**). The animal was placed on a wire grid lid and the lid was then closed to invert the mouse. The latency to fall from the lid (to the padded floor of the device 20 cm below) was recorded as the outcome measure.

Body Composition (DEXA)

Dual X-ray Absorptiometry (DEXA) measures fat percentage, lean mass, bone mineral density and bone mineral content (GE Lunar PixiMus I). We report only fat percentage and lean mass herein. (**Figure 2 C**)

Training Effect- Whole Body Training Physiology

We tracked the individual performance of the mice over the training period. *Training Force* (milliNewtons, mN), *Normalized Training Force* (mN/gbm, gbm=gram body mass), *Training Power* (milliWatts, mW), and *Normalized Training Power* (mW/gbm) were all tracked through the duration of the study. We report normalized training force as an affirmation of the progressive resistance aspect of the training protocol and normalized training power to assess relative intensity of exercise over time.

Training Force represented the total sum of force applied by the mouse while working out [equation: *Training Force = mass (mass of weight harness + mass of*

*mouse) * acceleration due to gravity (g)]*. *Normalized Training Force* divided training force by the mass of the mouse to give an indication of the quality of force produced (amount of force produced per gram of the mouse). *Training Power* represented the total amount of work done per second by the mouse while training {equation: Training Power (milliWatts, mW) = *Work (mJ, milliJoules) / seconds exercised [Work, or Training force (mN) * distance run (meters)]}. Normalized Training Power* was the training power performed per gram of body mass of the mouse, another measure of the quality of muscle performance.

Cellular Adaptations to Training

Muscle Function- Contractility

The specifics have been previously detailed (Graber, 2013; Graber, 2015 A). In brief: The extensor digitorum longus (EDL) and soleus (SOL) muscles were removed and then perfused in Krebs/Ringer buffer with 95% O_2/5% CO_2 at 25°C. A #4 gauge silk suture line was tied to the muscles at the myotendinous junctions, the muscle was then hung by the suture line from a force transducer (Aurora 300b) at the origin end and attached to a static clamp at the insertion end. The muscle was suspended between two platinum electrodes that were stimulated (Aurora High Power Bi-Phase Current Stimulator and a Dual-System Signal Interface, software: DMC v.4.1.6) under a variety of protocols to determine optimal length (L_0), maximum isometric contractile force (P_0), and velocity of contraction using the force clamp technique. Maximum unloaded velocity (V_{max})

was calculated using a derivation of the Hill Equation [$(V+b)(P/P_0 \pm a/P_0) = b(1+a/P_0)$, a and b are constants, V = max. velocity at fractional load] (Brooks & Faulkner, 1988). The data was curve fit (only $r^2>0.98$) using MatLab. Velocities of contraction at various percentages of P_0 were used to calculate power and the force-power curve. We report only the data from the SOL herein.

Muscle Hypertrophy

Soleus Wet Mass

Following *in vitro* physiology, the SOL was massed after being blotted dry.

Fiber Size-Histochemistry of Plantaris Muscle

We have previously published details of this method (Graber, 2014). Briefly: Super-cooled isopentane, and subsequently liquid nitrogen, were used to freeze isolated plantaris muscles. 10 micron thick sections cut on a cryostat were then stained using H&E (hematoxylin and eosin). Microscope images were photographed and circled with ImageJ software (NIH) to find the cross sectional area of fibers.

Anabolic Signaling

The level of phosphorylation in p70S6K (at the threonine 389 site) and Akt (at the threonine 308) was determined using a fairly standard Western Blot protocol previously detailed (Graber, 2015 B). We report the values from densitometry

analysis in the normalized format (all values normalized to the mean of the age-matched control). In the Supplement we normalize all values to the adult control to compare the phosphorylation states of the proteins in all four groups of mice. A brief description of the procedure follows:

Quadriceps femoris muscles were isolated and frozen in liquid nitrogen, and stored at -80° C. The muscles were homogenized in Mueller Buffer (Graber, 2015 B), which contains both protease and phosphatase inhibitors. The bicinchoninic acid kit (# 23225, ThermoScientific, Rockford IL) was used to determine protein concentration. Electrophoresis of both the AKT (Hoefer SE250, Holliston MA, water cooled, ~1.5 hours at 200 mV) and p70S6k blots (Biorad Mini-Protean,#165-8000, 45 minutes at 200 V) were on 10% acrylamide gels in Laemli buffer with the same blot control on every gel. Proteins on the gels were transferred to a PVDF membrane (blocked with 5% milk in TBS-T) using a Biorad semidry transfer (#170-3940), set at 14 V for 23 minutes with Bjerrum and Schafer-Nielsen transfer buffer. The Akt blot was first probed for p(308)-Akt, stripped and re-probed for Akt, then striped and reprobed for GAPDH. The p70S6K blots were handled in the same order. Lane control was accomplished using a 1:5000 dilution GAPDH antibody (Cell Signaling Technology #14C10). Akt, p(threonine308)-Akt, p70S6K, and p(threonine 389)-p70S6k were from Cell Signaling Technology, product #9272, #2965, #9202, #9205, respectively, with dilutions of 1:1000, 1:500, 1:1000 and 1:500 in % BSA/TBS-T with1% goat

serum; all incubated overnight at 4°C. All blots used a goat anti-rabbit horse radish peroxidase conjugated secondary antibody (Cell Signaling Technology #7074s) incubated for 1hour at room temperature, Western Dura-Signal Plus (ThermoScientific) as the substrate (5 minutes duration), were imaged using a Biorad Chemidoc XRS and densitometry analyzed using QuantityOne (Biorad).

Statistics

Data is shown as means ± standard error, as appropriate. Repeated measures general linear model (compare within subject in the rotarod and grip test), Student's t-tests (independent and paired), Student's T-Test, 2x2 ANOVA, ANCOVA use and report $\alpha<0.05$ as significant and $\alpha<0.10$ as showing strong evidence to refute the null hypothesis and representing a trend. For post-hoc testing LSD (Least Significant Difference) was used. To determine the relationship between two variables the linear regression analysis was used ($p<0.05$=significant). Comparison of linear regressions was done using the General Linear Model (GLM). Distributions of Plantaris fiber cross-sectional area was compared with independent samples median test and the 2-sample Kolmogorov-Smirnov. Direct comparisons of data from previous work (Graber, 2015 B) to the data from the current study using 2-way ANOVA and ANCOVA is reported in the supplement, but discussed in the Discussion section. This same data also is used for secondary analysis in Table 3 and discussed in the main

paper. Symbols: "*" denoted $p<0.05$ (significant) and "#" denoted $p<0.10$ (trend). IBM SPSS v20 was used for the analysis.

Results

Table 1 reports the results of repeated measurements and their absolute values. **Table 2** reports the results of the [non-repeated measures] outcome measurements. The 2x2 ANOVA and ANCOVA comparisons of the main outcome measurements between the adult and old groups is detailed in the Supplemental Section, along with the appropriate figures and tables, and briefly discussed in the Discussion section in the main paper and p-values for main effects of age, training and the age-training interaction on **Table 3**. The main paper reports the results of the old mice exclusively, except in the case of parameters that were not reported previously (in Graber, 2015 B), with the exception of **Table 3**, which details the comparison of the outcomes in both adult (previously reported) and old exercise groups (percent difference between age-matched control and exercise groups, and the results of the 2x2 ANOVAs/ANCOVAs--detailed in the Supplement), to examine for evidence of possible anabolic resistance.

WHOLE BODY TRAINING ADAPTATIONS

Animal Performance

Rota-Rod (**Figure 3, Table 1 and Table 2**)

Rotarod time difference in seconds (s), -17.7 s, between the two old groups was not statistically different (old exercise +8.67±7.68 s; old control -9.05±10.85 s; t=-1.28, p=0.218) (**Figure 3 A**). The percentage change with training in the older

groups was not significantly different (t=-0.942, p=0.359) (**Figure 3 B**). There was a wide data spread, however, with a few high-performing control mice driving the mean upward. Likewise, one low performing exercise mouse reduced the mean. There was no correlation of rotarod difference with either mass at sac (R=0.700, p=0.768) or initial mass (R=0.141, p=0.554). Mass was also not a significant predictor of percentage change (R=0.105, p=0.660).

Inverted Cling Grip Test (**Figure 4, Table 1 and Table 2**)

Grip time difference in seconds (**Figure 3 C**) between the two old groups was not statistically different (old training +52%) [t=-0.782, p=0.444], but there was significant increase from the first grip test to the post-intervention test within the old groups with control mice function gaining a mean of 20% and the exercised mice gaining 55%, though the gains were not statistically different between groups (2x2 Repeated Measures, F=4.936, p=0.039; but no significant interaction of grip with training status, p=0.444). Grip percentage change was not statistically improved in the old control mice (-22±11.9%) compared to in the exercised mice (+5±18.7) (**Figure 3 D)**. Body mass was not a significant predictor of grip test difference in the older mice.

Body Composition (Fat Percentage) - DEXA (Figure 4, Table 1)

Body mass was not different in the old control and exercise groups either before or after the intervention period. Body composition was significantly improved by

the training intervention with a -19.5±5.5% change in fat percentage in the elderly exercise group compared to old control, -0.4±8.1%, and adult control, +58±8.3%. Adult exercise mice were unfortunately not tested (DEXA not available at training initiation). Lean mass was not different between the groups. The statistics from this section were: 1-way ANOVA to compare %Change in fat %: f= 24.403, p<0.001. AC>OC, p<.001; AC>OE, p<.001; OC>OE, p=.072.

Training Effect- Whole Body Training Physiology

Normalized Training Force (mN/gbm)

We report only normalized force and power, since the non-normalized values revealed no additional information. As would be expected, the old exercise groups improved normalized force output over the training sessions. **(Figure 5 A).**

Training Velocity (m/s)

We measured the velocity (to derive power) maintained by both the adult and old mice during their exercise sessions. Since the mean training velocity was not previously reported (in Graber, 2015 B), we report it here. The mean training velocity maintained by the adult exercise group (0.092±0.00181 m/s) was 158% faster over the training sessions than the old group (0.058±0.00498 m/s) (Student's T-test, t=13.3, p<0.001).

Normalized Power (mW/gbm)

Normalized training power (**Figure 5 B**) increased over the training period for the old exercise mice, but only if the exceptional influence of a single outlier is not removed. The session number 36 group power (mean 30.39±8.16 mW) and normalized power (mean 0.885±0.23 mW/gbm) were driven by a single very high performing mouse (78.7 mW, 2.25 mW/gbm). This mouse was a definite outlier, considering the standard deviation (power SD 21.6 mW, and normalized power SD 0.61 mW/gbm), being 2.24 SD away from the mean. If the data is re-analyzed excluding this outlier, the session number 36 old group power (mean 22.3±1.51 mW) and normalized power (mean 0.657±0.037 mW/gbm) result in very different outcome measurements. The regressions become non-significant for both power and normalized power with respect to training session ($R=0.111$, $p=0.794$; and $R=0.149$, $p=0.795$, respectively). We present the data in Figure 5 B including the outlier, but denote normalized power in the older exercise mice as being questionably significant on Table 3 because of the undue influence of the outlier.

TISSUE and CELLULAR RESPONSE to TRAINING

Muscle Function- Contractility

SOL Maximum Force (P_0) and Normalized Force (P_0/gbm)

P_0 was not different with training (**Figure 6 A**) [control 178.2±9.7 mN; exercise 193.1± 7.3 mN; t=-1.153, p=0.263]. Normalized P_0 was also not different (**Figure 6 B**) [control=5.4±0.3 mN/gbm; exercise=5.8±0.3 mN/gbm; t=-0.922, p=0.368].

SOL Force-Velocity Curve and a/P_0

a/P_0 was reduced in the old exercise (0.026±0.001) compared to the control (0.031±0.002) old groups, indicating an alteration in the force-velocity curve (Student's T-test, p=0.059). However, none of the individual velocities (ranging from V_{max} to 90%P_0) were significantly different between the old exercise and old control groups (**Table S2 and Figure 7 A**).

SOL Power Production, P_{max}, and %P_0@P_{max}

There was no evidence that training improved power output in the old mice when the power output at 10%-90%P_0, P_{max} and %P_0@P_{max} were compared individually (**Table S3**). The force-power curve is shown on **Figure 7 B.**

Muscle Hypertrophy

SOL Wet Mass

SOL mass increased +15% with exercise in the older mice (Student's T-test, p=0.089) (**Figure 8 A**). The mean normalized SOL mass (g/gbm) was not significantly larger (+15%) in the old exercise animals compared to the controls (**Figure 8 B**). SOL fiber length had a small (8%) but significant increase (Student's T-test, p=0.04). Sol PCSA was not significantly increased after training.

Plantaris Muscle Fiber Hypertrophy

There was no significant change in plantaris muscle fiber CSA with training, when examined using the means of the CSA for each (**Figure 9 A**). There was large individual variability and a low number of subjects (n=4 OC; n=5 OE). However, as previously reported, the effect size of 0.80 in the adult group suggested that there was a change from the intervention, which may have not been detected because of the high variation (type 2 error, false negative). We had thus examined the distribution of the 2 adult groups in detail and reported in our previous work that there was a significant increase in the mean, median and overall distribution (rightward shift) in the exercise adult group compared to the control adult (**Figure S10**, also reported in Graber, 2015 B). Therefore, in the current study we also examined the pooled fiber data of the old groups (**Figure 9**

B). The old exercise mice (mean CSA, 1203.9±17.9 μm^2) were 4.8% larger than the old control (mean CSA, 1148.0±11.5 μm^2) (Student's T-test p=0.033).

We also compared the distributions of the CSA of the old control and old exercise groups (**Figure 9 C**). The distributions were different, with the control group skewed left. The median increased 9.6% with training (OC= 984.5, OE =1078.8). [Mann-Whitney U Test, p=0.043; Independent Samples Median Test, p=0.005]

Anabolic Signaling (Akt and p70S6K)

The relative densities of each mouse were normalized to the control mean (p70S6K values on **Table 2**; Akt values not shown).

p70S6K

There was a 32% treatment-related increase with training in the relative amount of p70S6K, p=0.032 (**Figure 10 A**). The phosphorylation (p-p70S6K) at the Threonine (THR) 389 position (THR389) was dependent upon training with exercise group having relatively more (+55%) phosphorylated protein than the control, p=0.005 (**Figure 10 B**).The ratio of p-p70S6K/p70S6K also had a significant 19% increase in the exercised group (p=0.005). Representative blots shown in **Figure 10 C**.

Akt

There was no difference in Akt (p=0.765), p(THR308)-Akt (p=0.267), or in the ratio of p-Akt/Akt, (p=0.305) (no figure representation, data not shown).

Discussion

Our weight training protocol was previously validated as a mimetic of human weight training for the mouse (Graber, 2015 B). The adult trained mice demonstrated evidence of improvement in all measured areas. In the current study we applied the training protocol to a cohort of elderly mice to determine the effect of training in aged animals. Our hypothesis was that we would see multiple areas of improvement with training, but that the effect would be modulated by the age of the mice. In other words, we expected to see evidence of resistance to anabolic stimuli manifested in a reduced or non-response in some of the outcome measures, particularly when compared to the response of the adult mice from the prior study. Indeed, there was a lessened effect in some of the measures that was sometimes obscured by high individual variability in the t-tests, hence in the Supplement we compare the means of all four groups of mice (adult control, adult exercise, old control and old exercise) using 2x2 ANOVA or ANCOVA, which has more power to detect change. Figures detailing the results of the Supplement are also included. **Table 3** highlights the adaptations from training experienced by both ages of trained mice. While it is clear that both ages of mice received benefit from the exercise protocol, the overall effect was lessened in the older cohort.

Anabolic resistance

The aged mice had less of a response to the training than the adult mice, possibly due to age-related anabolic resistance. **Table 3** details the results of the older exercise group when compared to the adult exercise group. In most cases (11 of 17) the adult mice experience a greater percentage difference in comparison to the age-matched control, and in many more cases this difference is significant (14 significant changes in AE versus only 6 for OE). Furthermore, the adult mice changes are directly improved in comparison to the old mice in __ of 17 measurements. 2X2 ANOVA and ANCOVA results of a head to head comparison of all 4 groups of mice are detailed in the Supplement, but the main effects are listed on **Table 3**. Though high variability may obscure some of the differences between the old and adult groups, there was a main effect of age for most relevant comparisons (11 of 15), other than the functional measurements and a couple of others (discussed below), indicating age associated dysfunction. Training was a main effect in 13 of 15 measurements indicating that training resulted in improvements. Only contractile velocity and power production had significant interaction of age*training that would indicate training was affected by the age of the mouse and vice versa. In total, Table 3 presents a compelling case that the older mice did experience a degree of anabolic resistance, the extent of which may have been possibly obscured by a high level of individual variability.

Anabolic resistance is defined as a reduced capacity for an organism to respond to stimuli that would generally result in an increase of protein synthesis. An age-associated decline in the ability to respond to anabolic stimuli has been chronicled with both nutrient sensing pathways and exercise (Burd, 2013; Fry, 2011; Koopman, 2009; Kumar, 2009; Degens, 2003). It has been suggested recently that the elderly possess potentially equivalent response mechanisms but may require a higher signal level to achieve the same benefit as younger adults (Symons, 2011; Moore, 2015; Bickel, 2011).

Possible Mechanisms Contributing to Anabolic Resistance

The systemic physiological environment may be less conducive towards muscle hypertrophy in the elderly, thus contributing to anabolic resistance. A good example is age-related global inflammation, which contributes to reduced exercise response (Merritt, 2013). Furthermore, multiple changes occur with age in the endocrine system including a global reduction in free testosterone and somatotropin, each being important anabolic hormones (Sipila, 2013; Valenti, 2010; Gray, 1991; Van den Beld, 2000). At the cellular level, impaired protein synthesis signaling with age has been implicated as a possible source of anabolic resistance (Fry, 2011 B). There is evidence that activation of mTORC1, the primary protein synthesis pathway (Glass, 2010), by resistance training is compromised in aging (Fry, 2011 A). In addition, nutrient signaling pathways involving amino acid transporters have recently been implicated in reduced

anabolic response (by mTOR activation) to exercise (Dickenson, 2013). Another example would be that the ability of the satellite cell to activate, proliferate, differentiate, and ultimately fuse with existing fibers to promote muscle repair and/or to contribute to hypertrophy is also compromised with age (Conboy, 2005; Gopinath, 2008). Thus, anabolic resistance is multi-etiological, as is sarcopenia, and thus multiple strategies may be needed to improve response to anabolic stimuli in the elderly.

By utilizing multiple combinations of interventions acting upon different mechanisms of anabolic resistance, sarcopenia may be more treatable. Resistance training in combination with nutritional and ergogenic support may be a more potent anabolic agent than any single therapy alone (Devries, 2014; Forbes, 2012; Candow, 2012). Our working hypothesis was that impaired anabolic signaling pathways contribute to age-associated anabolic resistance, whether the impaired arises from increased noise or from a dysfunctional systemic environment.

Evidence of Anabolic Resistance in the Current Study

At the level of the whole organism we examined functional performance, body composition and training physiology. The rotarod (overall neuromuscular motor function) and inverted cling grip test (strength/endurance) were used to assess functional performance. In the both measurements the old mice showed some

evidence of improvement (main effect of training, see **Table 3** and the supplement, **Figures S1 and S2**) to level statistically indistinguishable from the adult exercise group. However, when the older mice groups were analyzed alone there was little evidence of improvement as a result of training (**Table 1 and 2**), though there was some evidence that the control mice performed worse in the grip test than the exercise group after the intervention period (**Table 1**). Therefore we conclude that elderly animals can improve functionally to a similar extent as do adults. This has been evidenced in human studies of exercise and function as well (Fragala, 2014; Seynnes, 2004). In previous work (Graber, 2014), we demonstrated that voluntary wheel running resulted in similar adaptations of increase rotarod ability in both adult and old mice. Since functional ability can benefit from neurological adaptations, improvements in balance and coordination, and other non-muscular and non-anabolic processes, the similar level of adaptation in both age groups does not necessarily reflect a lack of anabolic resistance. It is entirely possible, and deserving of future study, that the adult animals gained more functional ability from increases in contraction while the older mice gained more ability from increases in the more neurological aspects of the training.

The exercised mice improved in body composition, consistent with human literature (Peterson, 2011). Unfortunately we cannot compare the level of improvement to the adult exercise mice, since a DEXA machine was not

available to measure that group. However, the training physiology measurements do show evidence of blunted exercise response with training force increases during training lessened and the mean training velocity lower in the old animals (**Figure S3 A**). Training power increases were lower in the old mice when compared to the adults (**Figure S3 B and C**), but, perhaps more importantly, if the outlier is removed, training power did not increase at all in the older cohort.

At the cellular and tissue level, there was additional evidence of a blunted response to the exercise stimulus. SOL *in vitro* contractile physiology measured absolute isometric force, contractile velocity, and power production (**Figures S4, S5 and S6**). P_0 did not increase statistically in either the adult or old groups (**Figure S4**). However, when the soleus P_0 was normalized to body mass, there was a main training effect giving credence to an improved response overall, though response in the older mice was blunted in comparison to the adults group (**Figure S4**). Normalizing soleus force to body mass is important because a linear relationship exists between the two (soleus being a postural muscle).

There were no statistical increases in isometric force, contractile velocity or power production in the older mice. The adult mice, however, experienced increases in both velocity and power at higher percentages of P_0 (**Figures S5 and S6**). There was, however, a main effect of training that gave some evidence to a limited increase in velocity and in power, particularly at higher percentages

of P_0, which we have previously found to be increasingly (compared to lower $\%P_0$) impaired in older mice (Graber, 2015 A).

Hypertrophy was measured using the wet mass of the SOL and the CSA of plantaris fibers. The soleus increased in mass to a similar extent in the older mice when comparing exercise to control (**Figure S7**). However, the length of the SOL fibers was increased to a greater proportion (though not significant) in the older mice (**Table 3**). Thus the SOL grew by adding serial sarcomeric units and force output is increased by increasing the diameter of the sarcomeres, not the length. This might be reflected in the lack of significant contractile function improvement in the older mice despite hypertrophy. The plantaris fibers demonstrated small increases in size with the adults gaining slightly more (though the differences were not significant), though the median shift was slightly larger in the older mice (**Figure S8**). The results are consistent with the prevailing exercise literature. Compared to young controls, older subjects have demonstrated a reduced capacity to increase muscular strength in human training studies (Kosek, 2006) as well in some animal models of hypertrophy such as synergistic ablation (Blough, 2000). If given adequate stimulus older muscle can hypertrophy to a similar extent (percentage increase) as younger (Thompson, 1994).

Surprisingly, we found an increased signaling response to exercise when measured by the phosphorylation level of p70s6k in comparison to the age-matched control (+30% AE, versus +55%, OE) (**Table 3**). However, because the absolute levels of response compared to the adult control were similar, the massive increase may be due to the much lower basal level of activity in the old control mice (**Figure S9**).

Conclusion

Both the older and adult mice benefitted by undergoing positive adaptations to the training stimulus. However, the older mice experienced a blunted response in some of the measurements, evidence of anabolic resistance. Surprisingly the older mice had a high level of p70s6k phosphorylation, indicating an upregulated level of protein translation, in comparison to the age-matched controls. We hypothesize that since training greatly increased mTORC1 signaling response in the older animals, extending the training time might allow the older mice to receive similar benefits as the adult mice achieved in the shorter time period. It would be interesting to directly measure protein fractional synthesis rates of the mice using tracers before and after the training period to assess if the signaling changes correlate directly to increases in protein production. Thus, it is likely that the older mice may have needed more training time to improve to a similar level as their younger counterparts. Our future goals include investigating the synergy of additional intervention strategies, such as nutritional supplementation of

branched chain amino acids, with resistance training to determine if the age-blunted response can be modulated.

Acknowledgements:

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For Technical Assistance: Katie Fandrey (Training Effect Physiology); Janice Shoeman, Rachel Borgstahl, Windy Torgerud, Alicia Behr, Nathan Proft, Steven Quiring, and Amanda Richabaught (histology/CSA**)**, Haiming Liu (for assistance during tissue collection), and JingYing Zhang (for assistance with some Western Blots and tissue homogenization).

Table Legends

Table 1 Repeated Measurements of Absolute Values Grip test was significantly reduced in the control mice and fat% was significantly reduced in the exercise mice after the training period. No other repeated measures were significantly changed. **Symbols and Abbreviations:** OC=old control, OE=old exercise; 1=pre-intervention measurement, 2=post-intervention measurement; p-val=p-value from paired t-test (significance in **bold**); mass (either pre- or post-intervention) was not a significant adjustor for either grip or rotarod.

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Figure 9 Plantaris CSA A. Means by Mouse OC, n=4; OE, n=4 **B. Pooled Fibers by Group** Mean is +5% with exercise. OC, n=3173; OE, n=1524 **C**. **Histogram Distribution of the frequency percentage of CSA** The median was 10% larger in the exercise group and there was a significant shift rightwards in the distribution of the exercise group CSA, indicating that there were more larger fibers in the exercise group than in the control group. **Notes:** OC=old control,

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measure	unit	OC 1	OC 2	p-val	OE 1	OE 2	p-val
grip	s	97.1±15.2	64.8±8.8	**0.023**	116.1±27.7	100.6±20.7	0.434
rotarod	s	95.8±9.4	86.8±4.5	0.424	83.2±6.1	91.9±9.0	0.292
Lean m	g	24.7±0.6	24.7±0.4	0.930	25.8±0.7	25.2±0.9	0.511
fat	%	17.8±1.2	17.2±1.4	0.654	18.3±1.7	14.6±1.5	**0.013**
mass	g	32.7±0.6	32.3±1.0	0.589	34.0±0.9	32.3±1.5	0.298

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Measurement	Unit	Control n=12	Exercise n=9	Percent Dif.	Effect Size	p-value
Body Mass Ini.	g	32.7±0.7	34.0±0.8	+4	**-**	0.237
Body Mass Sac.	g	32.3±0.6	32.9±0.9	+2	0.15	0.770
BMI at sac.	kg/m^2	3.3±0.1	3.5±0.1	+6	0.68	0.157
Fat% Change	%	-0.4±8.3	-19.5±5.5	-480	1.15	**0.072**
Rotarod Time Dif.	s	-9.1±10.9	+8.7±7.7	+196	0.77	0.218
Grip Test Time Dif.	s	-32.0±12.2	-15.5±18.8	+52	0.52	0.444
SOL Mass	mg	9.5±0.6	10.9±0.5	+15	0.79	**0.089**
SOL Mass/gbm	mg/gbm	0.296±0.019	0.342±0.031	+7	0.69	0.213
SOL Length	mm	11.16±0.26	11.94±0.21	+7	3.75	**0.040**
SOL Fiber Length	mm	7.8±0.2	8.4±0.1	+8	0.97	**0.04**
SOL PCSA	mm^2	0.80±0.04	0.96±0.04	+20	0.12	0.321
SOL P_0	mN	178.2±9.7	184.6±7.3	+4	0.22	0.263
SOL P_0/gbm	mN/gbm	5.4±0.3	5.8±0.3	+8	0.02	0.368
a/P_0	mN^{-1}	0.031±0.002	0.026±0.001	-16	0.32	**0.059**
Plant. Median CSA	μm^2	984.5	1078.8	+10	n/a	**0.005**
Quad p-p70S6K	nrd	1.000±0.122	1.546±0.129	+55	1.50	**0.009**
Quad p70S6K	nrd	1.000±0.090	1.319±0.081	+32	1.18	**0.032**
Quad p-p70/p70	nrd	1.000±0.005	1.186±0.006	+19	1.72	**0.005**

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Measurement	AE n=6	OE n=9	p-value	ME Age	ME Training	ME INT.
Rotarod Time Dif.	*+127*	(+196)	0.560	0.395	**0.026**	0.509
Grip Test Time Dif.	*+81*	(+52)	0.650	0.992	*0.080*	0.556
N. Tr. Force	↑	↑	**<0.001ł**	N/A	N/A	N/A
N. Tr. Power	↑	?	**<0.001ł**	N/A	N/A	N/A
Final Body Mass	(-9)	(+2)	*0.090*	**0.001**	0.359	0.197
SOL Mass	**+15**	*+15*	0.996	**<0.001**	**0.016**	0.134
N. SOL Mass	**+26**	(+15)	0.410	0.160	**0.009**	0.465
SOL Fiber Length	**+6**	**+8**	0.567	*0.093*	**0.020**	0.771
SOL PCSA	(+10)	(+20)	0.792	**<0.001**	*0.088*	0.661
SOL P_0	(+1)	(+4)	0.111	**<0.001**	0.440	0.583
N. SOL P_0	*+11*	(+8)	0.577	**<0.001**	*0.071*	0.473
a/P_0	**-22**	*-16*	0.359	**<0.001**	**0.008**	0.987
Velocity	↑	-	**<0.05**	**All**	*@ 50*,**60,and 80%P_0**	*@ 80 and 90%P_0*
Power	↑	-	**<0.05**	**All**	*@ 60*, **80 and** *90***%P_0**	*@ 90%P_0*
%P_0 @ P_{max}	**+7**	(+2)	**0.043**	0.188	**0.022**	0.188
Plant. Mean CSA	**+5.4**	**+4.8**	0.500	**0.001**	**0.001**	0.172
Quad p-p70s6k	**+30**	**+55**	0.184	**0.042**	**0.044**	0.939
Total Significance	**14**	**6**	**6**	**11**	**13**	**2**

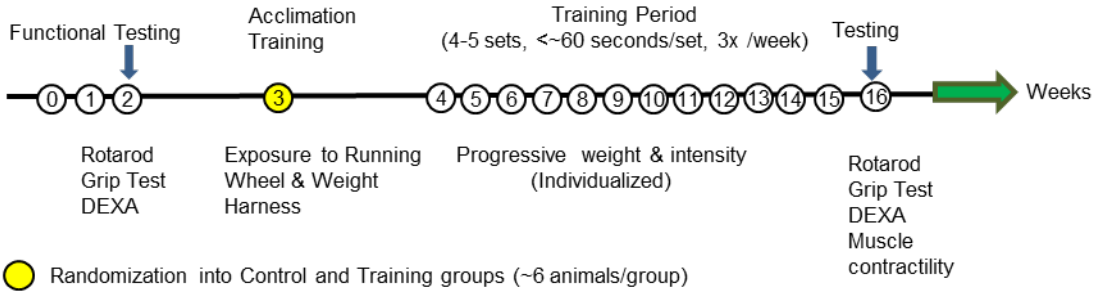
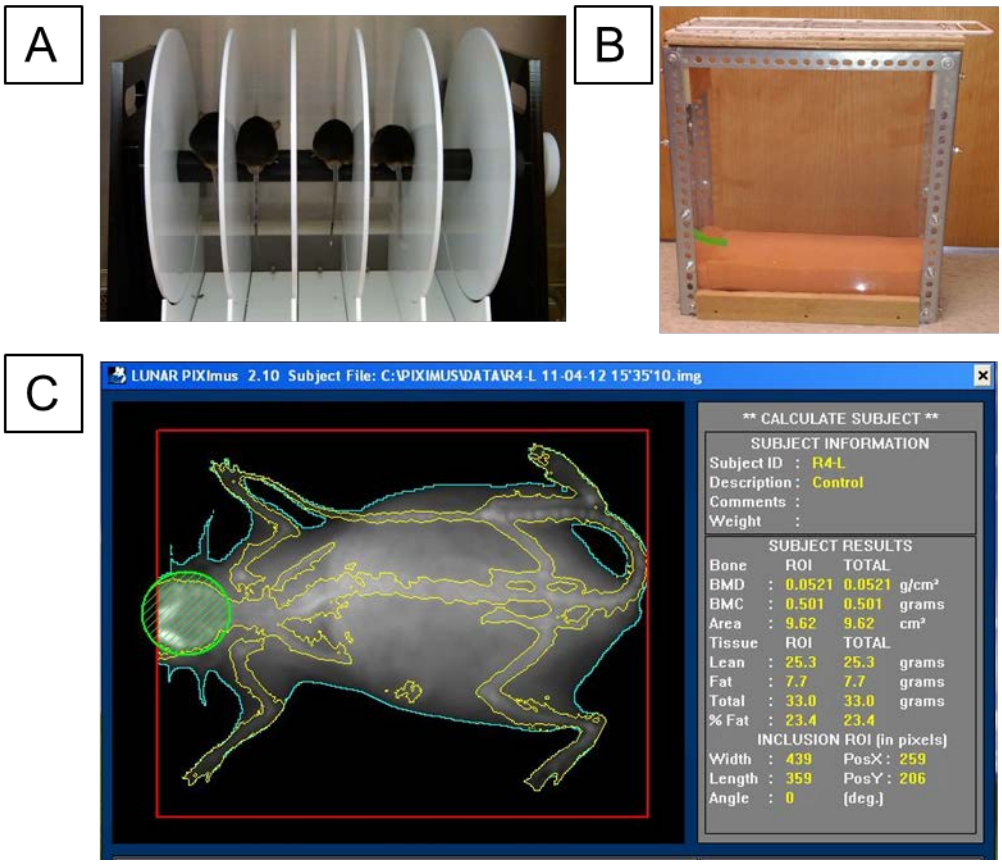


Figure 1 Research Design



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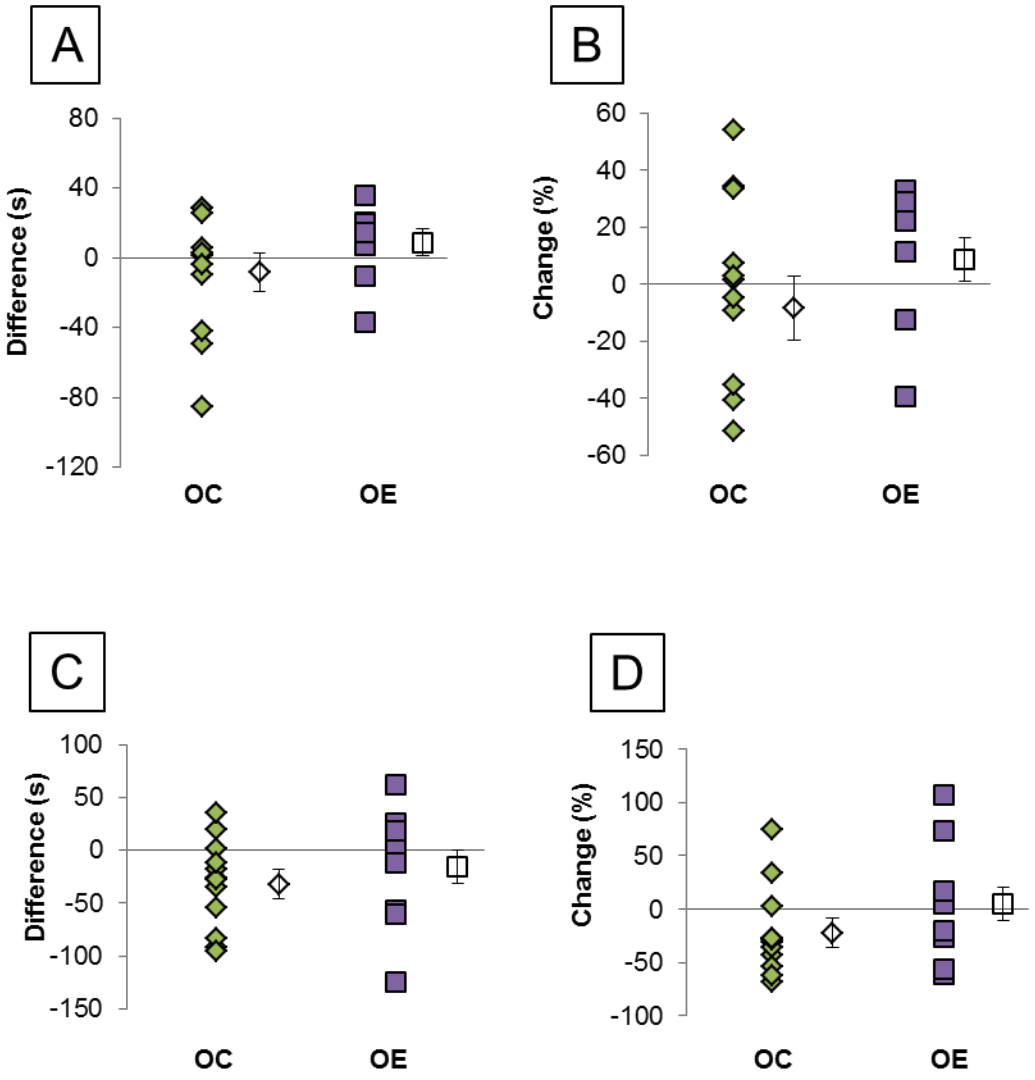

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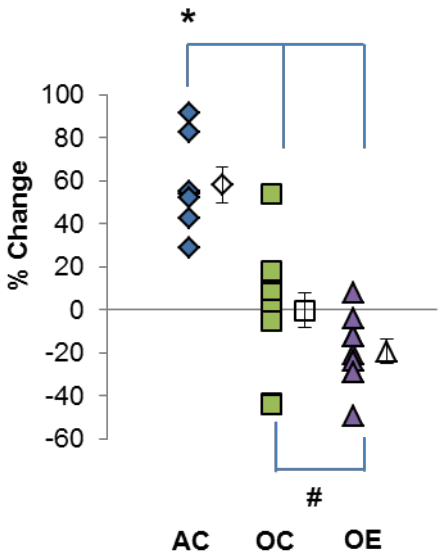

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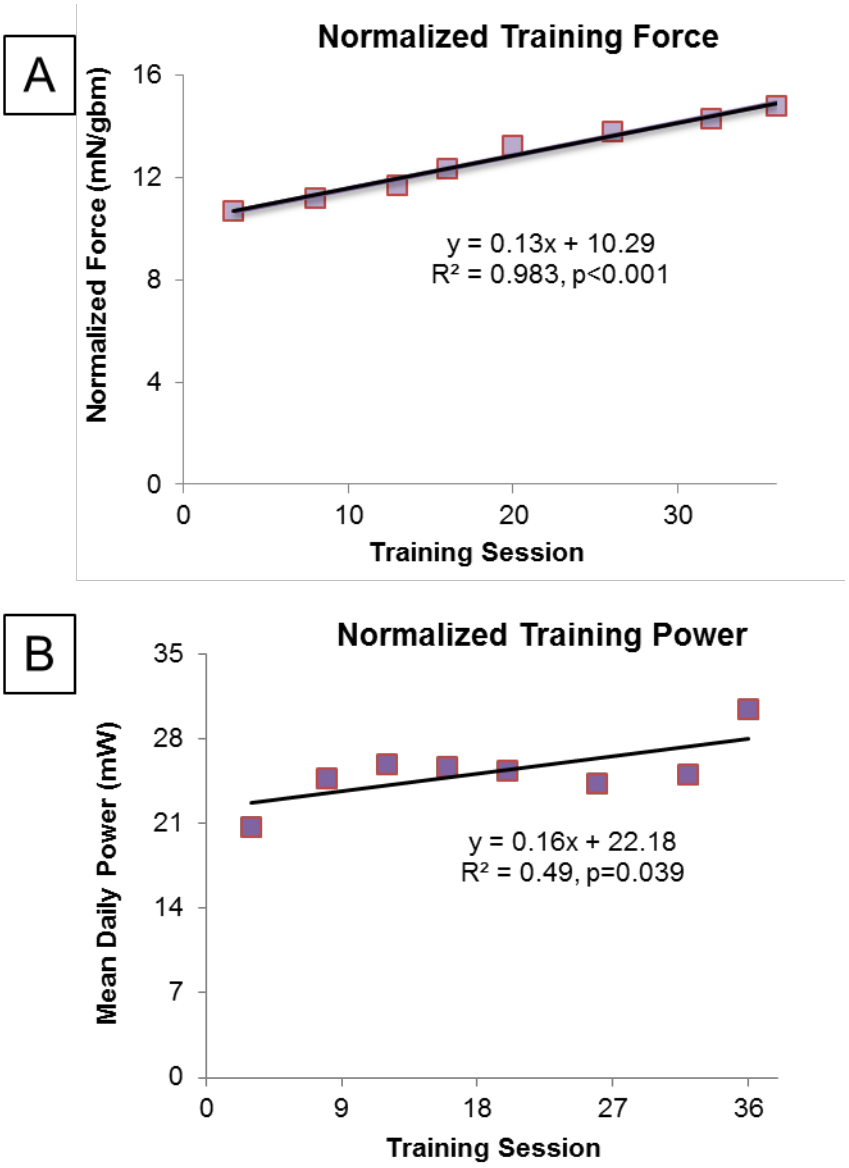

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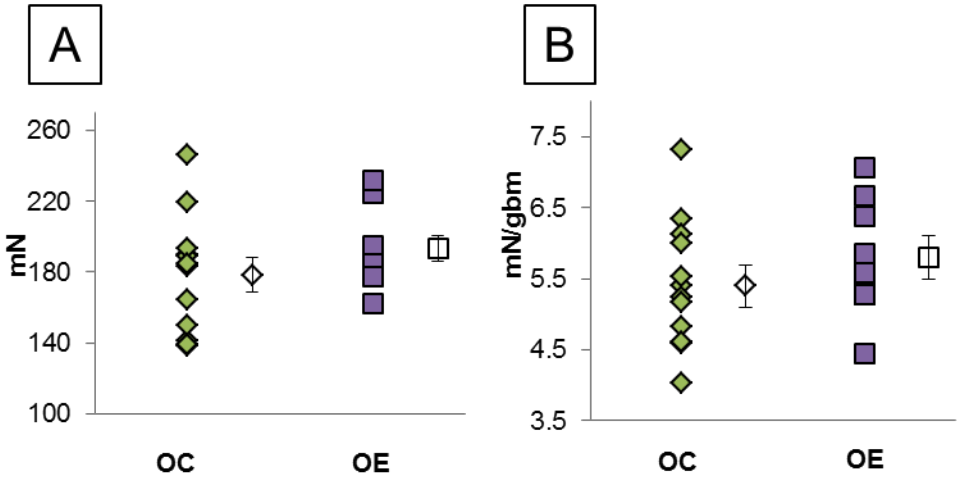

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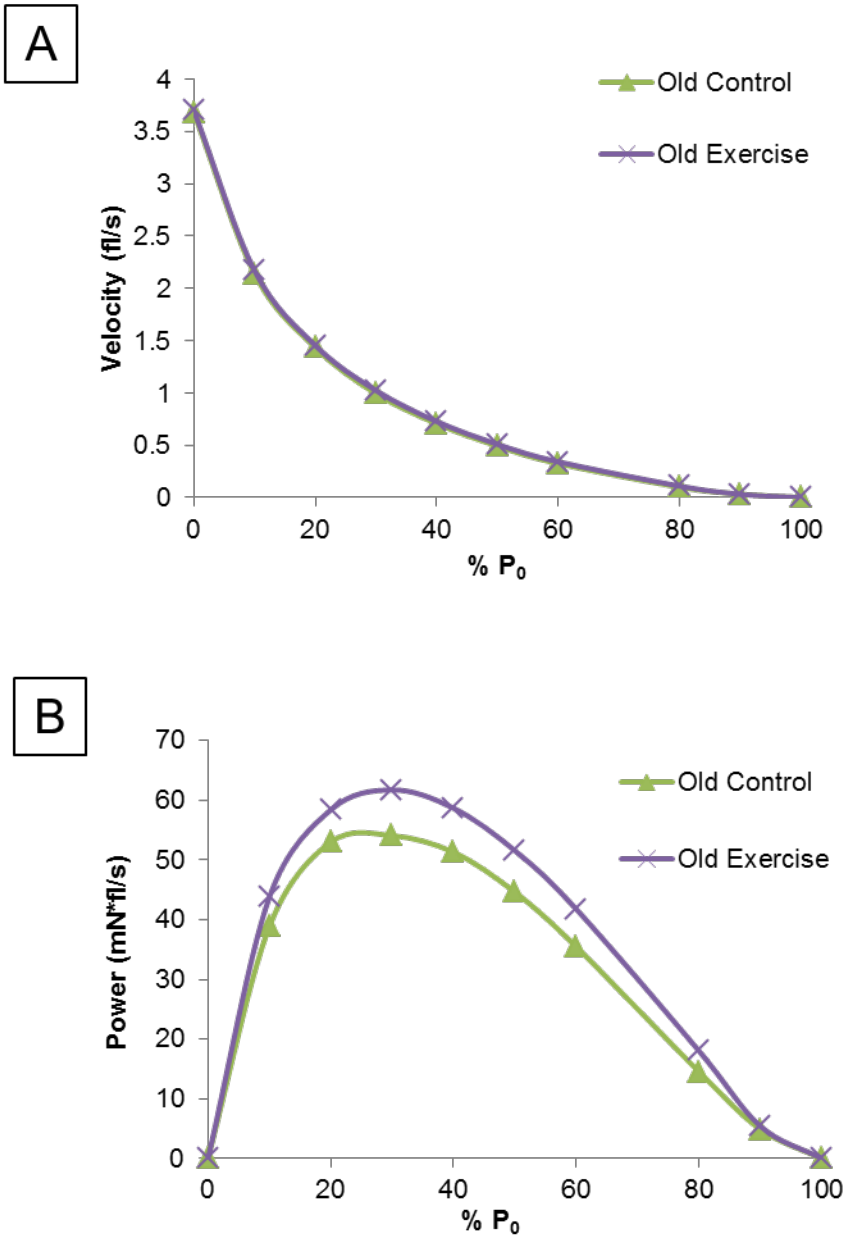

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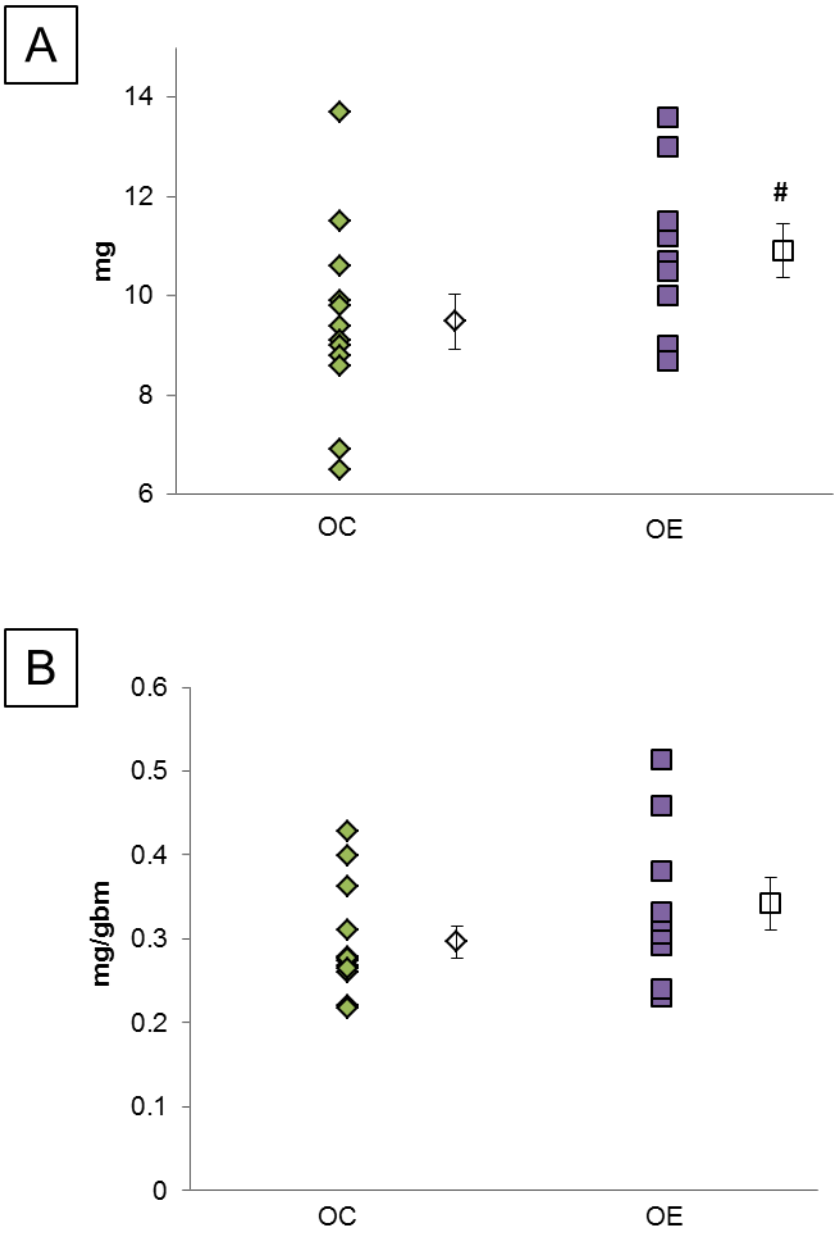

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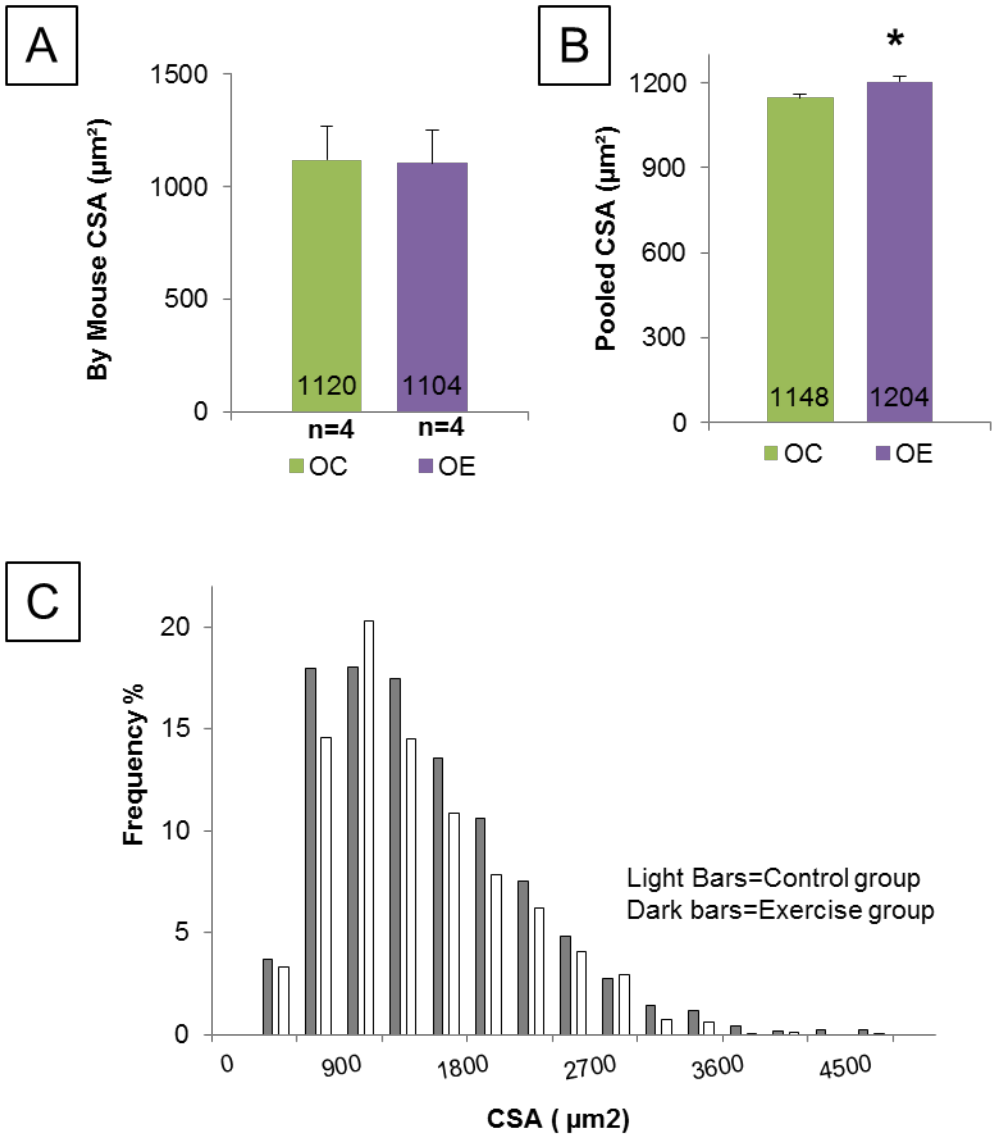

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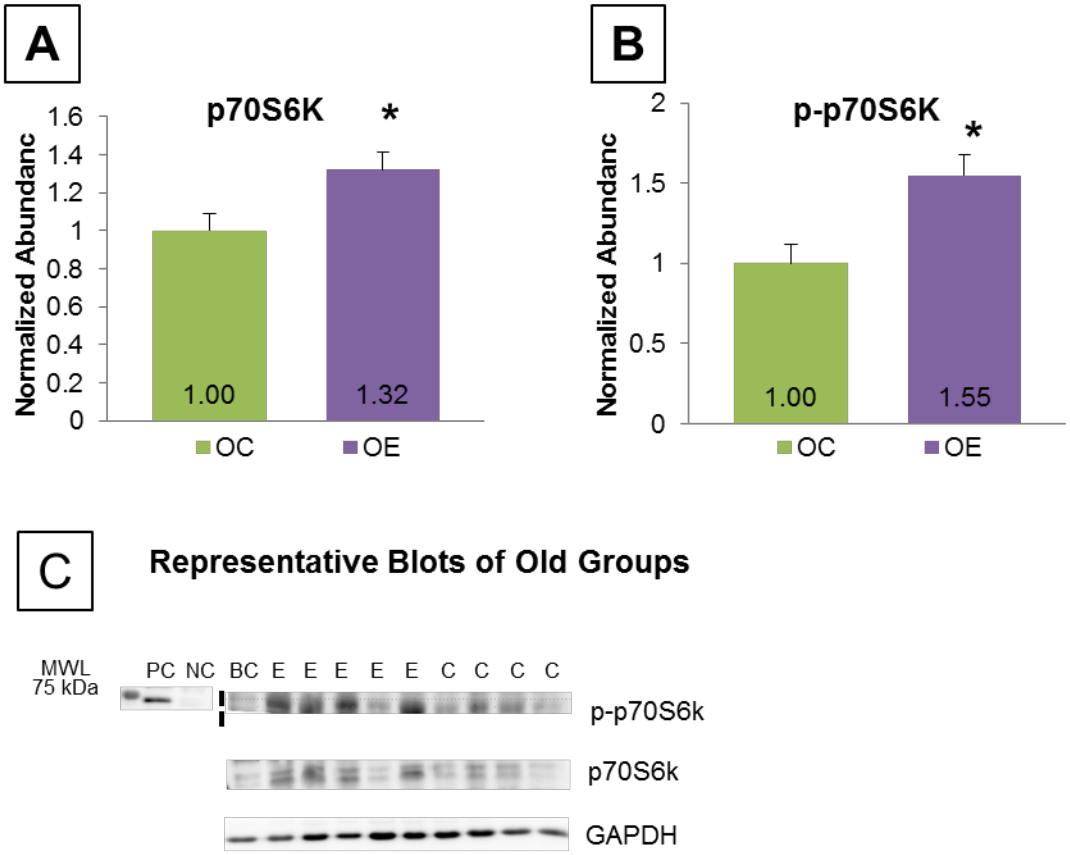

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Supplemental Section Results of Side by Side Analysis of the Adult Exercise and Adult Control Groups (previously reported in Graber, 2015 B) with the Old Exercise and Old Control Groups.

<u>Note:</u> Statistical comparisons will be detailed under the appropriate section in brackets.

Table S1 details animal characteristics of body mass, changes in body mass, muscle mass, P_0, P_{max}, V_{max} and p-values of the main effects of a 2x2 ANOVA (2 ages and 2 treatment statuses) comparison of the adult mice detailed in Graber, 2014 B to the old mice, with LSD multiple-comparison posthoc tests represented by letters and symbols. The following convention will be used to denote the different groups (used interchangeably throughout): AC=adult control, AE=adult exercise, OC=old control, and OE=old exercise.

WHOLE BODY TRAINING ADAPTATIONS

<u>Animal Performance</u>

Rota-Rod (**Figure S1**)

This outcome tested overall motor function (gait speed, balance, coordination, stamina, power). After training the control mice had reduced function compared to exercise mice.

There was a significant main effect of training (+146%) when comparing all four groups (overall control -16.8±6.8 s, overall exercise +7.8±8.1 s), with no significance for age or age*training interaction (**Figure S1 A and B**).

[2x2 ANOVA F=2.327, p=0.093; Main Effects: Age, p=0.395; Training, p=0.026; age*training interaction: p=0.509; LSD Posthoc: AE>AC, p=0.055; AE=OC, p=0.326; AE=OE, p=0.900; AC=OC, p=0.240; OE>AC, p=0.023]

When all 4 groups were analyzed for percentage change, there was a main effect of training (+197%) (overall control -11.2±7.8%, overall exercise +10.9±9.3%, values adjusted for mass), with no significance for main effect of age or age*training interaction (**Figure S1 C**).

[2x2 ANCOVA, adjusted for body mass at sacrifice, F=2.181, p=0.094; Main Effects: Age, p=0.992; Training, p=0.080; age*training interaction: p=0.556; LSD Posthoc: AE=AC, p=0.129; AE=OC, p=0.250; AE=OE, p=0.696; AC=OC, p=0.711; OE=AC, p=0.265]

Simple linear regression of difference in seconds dependent upon mass at sac (R=0.325, p=0.053) was not statistical when all four groups were compared (**Figure S1 D**). There was also no correlation (R=0.065, p=0.703) with initial mass. Mass at sacrifice was correlated with rotarod percent change analyzed for all four groups.

[R=0.367, p=0.028, % = 75.791 – 2.185 (mass at sac in grams)]

Inverted Cling Grip Test (**Figure S2**)

This outcome measurement tested overall muscle strength and stamina. After training the control mice had reduced function compared to exercise mice.

There was a significant training effect (74.3% improvement) when comparing all 4 groups (**Figure S2 A and S2 B**), with no effect of age or age*training interaction.

[2x2 General Linear Model ANCOVA, adjusted for body mass at sac, F=4.230, p=0.008 Main Effects: Age, p=0.151; Training, p=0.025; age*training interaction, p=0.115; LSD Posthoc: AE (n=6) > AC (n=9) , p=.093; AC < OC (n=12) p=0.096; AC<OE (n=9) p=0.043; AE=OC, p=0.917; AE=OE, p=0.773]

Grip percentage change was not statistically improved when comparing all four groups (AC -51±13.5% and AE -19±20.5%) (**Figure S2 C**).

[2x2 ANOVA, F=2.756, p=0.107. Main Effects: Age, p=0.107; Training, p=0.075; age*training interaction: p=0.875]

When all four groups were analyzed together, body mass at sacrifice had a moderate Pearson correlation, R=-0.433, to the grip difference (in seconds) (**Figure S2 D**). Mass was not a significant predictor of the grip percent change (R=0.261, p=0.125). Initial body mass, was not correlated with either grip difference or percent change.

[Linear Regression, R=-0.433, p=.004, linear equation: grip difference (seconds) = 155.676 -5.844 (mass at sac in grams)]

Training Effect- Whole Body Training Physiology

Training Force (mN) and Normalized Training Force (mN/gbm)

As would be expected, both the old and the adult exercise groups increased normalized force output over the training sessions. The adults ultimately improved to a greater degree and at a faster rate in training force. **(Figure S4 A and B)**

[Training Force: slopes of the linear regressions were significantly different with the mean increase (adjusted) of 7.6% with training in the adult group; Univariate GLM ANCOVA, adjusted for training session, F=368, p<0.001; Main Effect of Age group, p<0.001, training session as adjustor, p<0.001]

Mean Training Velocity (m/s), Training Power (mW) and Normalized Training Power (mW/gbm)

The mean training velocity maintained by the adult exercise group (0.092±0.00181 m/s) was 158% faster over the training sessions than the old group (0.058±0.00498 m/s).

[Student's T-test, t=13.3, p<0.001]

Training power increased in both the adult and old exercised mice, although more so in the adult (**Figure S5 B**). The regression curve of training power in relation to training session number (**Figure S5 C**) were significantly different with a mean increase (adjusted) of 171% with training in the adult group (mean training power 43.1±0.98 mW) compared to the old (25.1±0.98 mW).

[Univariate GLM ANCOVA, adjusted for training session, F=93, p<0.001; Main Effect of Age group, p<0.001, training session as adjustor, p<0.001]

Normalized training power also increased over the training period for both the old and adult mice. The regressions were significantly different with the mean increase (adjusted) of 162% with training in the adult group (mean training power 1.19±0.028 mW/gbm) compared to the old (0.74±0.028 mW/gbm) (**Figure S5 D**).

[Univariate GLM ANCOVA, adjusted for training session, F=93, p<0.001; Main Effect of Age group, p<0.001, training session as adjustor, p<0.001]

IMPORTANT NOTE: The session number 36 old group training power (mean 30.39±8.16 mW) and normalized training power (mean 0.885±0.23 mW/gbm)

were driven by a single very high performing mouse (78.7 mW, 2.25 mW/gbm). This mouse was a definite outlier, considering the standard deviation (training power SD 21.6 mW, and normalized training power SD 0.61 mW/gbm), being 2.24 SD away from the mean. If the data is reanalyzed excluding this outlier, the session number 36 old group training power (mean 22.3±1.51 mW) and normalized training power (mean 0.657±0.037 mW/gbm) result in very different outcome measurements. The regressions become non-significant for both power and normalized power with respect to training session (R=0.111, p=0.794; and R=0.149, p=0.795, respectively).

TISSUE and CELLULAR RESPONSE to TRAINING

Muscle Function- Contractility

SOL Maximum Force (P_0) and Normalized Force (P_0/gbm)

When comparing all 4 groups there was an age main effect, 25% reduction in force with age (combined adult 248.2±8.4 mN; old 185.6±7.3 mN) (**Figure S6 A**). Neither the training effect nor the training*age interaction was significant. There was a significant moderate linear relationship (R=0.690, p<0.001) between body mass and P_0 (**Figure S6 C**).

[P_0: 2x2 ANOVA F=400, p<0.001; Main Effects: Age, p<0.001; Training, p=0.440; age*training interaction: p=0.583; LSD Posthoc: AE=AC, p=0.881; AE>OC, <0.001.; AE>OE, p=0.003; AC>OC, p<0.001; AC>OE, p=0.001]

Normalized force production was 39% higher in the adult compared to the old mice (main effect age) (**Figure S6 B**). There was also a main effect of training (+9.4%), but no significant interaction between age and training.

[Normalized P_0: 2x2 ANOVA F=396, p<0.001. Main Effects: Age, p=<0.001; Training, p=0.063; age*training interaction: p=0.408; LSD Posthoc: AE>AC, p=0.081; AE>OC, p=<0.001; AE>OE, p<0.001; AC>OC, p<0.001; AC>OE, p=0.002]

SOL Force-Velocity Curve and a/P_0 (Figure S7)

When analyzing all four groups there was a mean increase of 38% in a/P_0 with age and a mean decrease of 19% with training (main effects). There was an age effect, but no age-training interaction. The post hoc revealed that the old exercise group had a similar curve to the adult control group.

[a/P_0 2x2 ANOVA F=236, p<0.001. Main Effects: Age, p<0.001; Training, p=0.008; age*training interaction, p=0.987; LSD Posthoc: AE<AC, p=0.69; AE<OC, p<0.001; AE<OE, p=0.006; AC<OC, p=0.001; AC=OE, p=0.213; OE<OC, p=0.043]

None of the individual velocities (from V_{max} to 90%P_0) were significantly different between old exercise and old control groups. There were differences between the old and adult mice (**Table S2**, **Figure S7 A, B, and C**). Specifically, the main effect of age was significant (p<0.001) for all velocities V_{max} through 90%P_0 and there was evidence of the main effect of training at 50, 60, and 80%P_0 (p=0.098, 0.046 and 0.009, respectively) and the age*training interaction at 80 and 90%P_0 (p=0.087, 0.085 respectively) (**Figure S7 A, C,** and **Table S2**). Additionally, as was previously reported (Graber, 2015 B), there were differences with training between the adult groups.

[2x2 ANOVA with LSD posthoc testing, Details on **Table S1**]

SOL Power Production, P_{max}, and $\%P_0@P_{max}$

There was no evidence that training improved power output in the old mice when the power output at 10%-90%P_0, P_{max} and $\%P_0@P_{max}$ were compared individually (**Table S3**). However, overall, when comparing all four groups (**Table S3 and Figure S8**), there were age-related differences (main effect age, adult>old) at all parameters (10%-90%P_0, P_{max} and $\%P_0@P_{max}$) and main training effects (training>control) from 60-90%P_0 and $\%P_0@P_{max}$. The 90%P_0 power was the only value with evidence of an interaction (training*age, p=0.070) with the adult exercise group increasing 32% over the adult control, but there was no change in the old mice.

Muscle Hypertrophy

SOL Wet Mass (Figure S9 A and B)

SOL mass increased +15% with exercise in the older mice. [Student's T-test, p=0.089] When comparing all 4 groups, the main effects of age (+23% in adults), and training (+18%) were significant. There was no significant interaction (age*training). (**Figure S9 A**)

[2x2 ANOVA F=10.271, p<0.001 Main Effects: Age, p<0.001; Training, p=0.014; age*training interaction: p=0.617; LSD Posthoc: AE (n=6) Soleus 15% > AC (n=13) , p=.019; AC>OC (n=12) p=0.001; AC=OE (n=7) p=0.159; AE>OC, p<.001; AE>OE, p=0.002]

The mean normalized SOL mass (g/gbm) was not significantly larger (+7%) in the old exercise animals compared to the controls, but the 2X2 ANOVA revealed a significant training effect (+16.8% after training). There was no significant

interaction, but there was a trend in the main effect of age term (-8.7% in older mice) (**Figure S9 B**).

[2x2 ANOVA F=3.053, p=0.042 Main Effects: Age, p=0.077; Training, p=0.019; age*training interaction: p=0.279; LSD Posthoc: AE 26%>AC, p=0.021; AE>OC, p=0.007; AE>OE, p=.085; AC=OC, p=0.566; AC=OE, p=0.637]

Plantaris Muscle Fiber Hypertrophy

There was no significant change in plantaris muscle fiber CSA with training, but there was evidence of age-associated atrophy, when the 4 groups were examined using the means of the CSA for each mouse in the subsets measured (**Figure S10 A**). There was large individual variability and a low number of subjects.

However, the effect size of 0.80 in the adult group suggests that there was a large change from the intervention, which may have not been detected because of the high variation (type 2 error, false negative). We had thus examined the distribution of the 2 adult groups in detail and reported in our previous work that there was a significant increase in the mean, median and overall distribution (rightward shift) in the exercise adult group compared to the control adult (**Figure S10 C**, also reported in Chapter 3). Therefore, in the current study we also examined the pooled fiber data of all four groups (**Figure S10 B**). The adult exercise mice CSA was 5.4% larger than the adult control and the old exercise mice (mean CSA, 1203.9±17.9 μm^2) were 4.8% larger than the old control (mean

CSA, 1148.0±11.5 μm^2), with a +5% main effect of training. The old mice, overall, had fibers 40% smaller than the adult (main effect of aging).

[Pooled CSA 2x2 ANOVA F=742, p<0.001. Main Effects: Age, p<0.001; Training, p=0.001; age*training interaction: p=0.172; LSD Posthoc: AE>AC, p<0.001; AE>OC, p<0.001; AE>OE, p=0.001; AC>OC, p<0.001; AC>OE, p<0.001; OE>OC, p=0.033]

We also compared the distributions of the CSA of the old control and old exercise groups (**Figure S10 D**). The distributions were different, with the control group skewed left. The median increased 9.6% with training (OC= 984.5, OE =1078.8).

[Mann-Whitney U Test, p=0.043; Independent Samples Median Test, p=0.005]

Anabolic Signaling (Akt and p70S6k)

p70S6k

There were no treatment related changes in the relative amount of p70S6K, though there was an effect of age (-60% in the old) (**Figure S11 B**). However, the phosphorylation of (p-p70S6K) at the Threonine (THR) 389 position (THR389) was dependent upon training with the adult (+30%) and old (+55%) exercise groups having relatively more phosphorylated protein than the age-matched controls (**Figure S11 A**). In addition, there was an age effect with -18% phosphorylation of p70S6K overall in the older quadriceps. The ratio of p-p70S6K/p70S6K only showed a significant difference in comparison of the adult control to the old exercise group, with the old exercise group showing an increase of 43% (means: AC 1.00±0.16; AE 1.21±0.14; OC 1.21±0.05; OE 1.00±0.06). However, there was a main effect of training, +21%; and a main effect of age, +22% in the older mice.

[Normalized p70: 2x2 ANOVA F=5.94, p=0.004. Main Effects: Age, p=0.001; Training, p=0.199; age*training interaction: p=0.665; LSD Posthoc: AE=AC, p=0.572; AE>OC, p=0.001; AE>OE, p=0.020; AC>OC, p=0.004; AC>OE, p=0.058, OE=OC, p=0.185]

[Normalized p-p70: 2x2 ANOVA F=6.03, p=0.004. Main Effects: Age, p=0.029; Training, p=0.002; age*training interaction: p=0.629; LSD Posthoc: AE>AC, p=0.056; AE>OC, p=0.001; AE=OE, p=0.210; AC>OC, p<0.055; AC=OE, p=0.377, OE>OC, p=0.005]

[Normalized p-p70/p70 ratio: 2x2 ANOVA F=3.408, p=0.035. Main Effects: Age, p=0.042; Training, p=0.044; age*training interaction: p=0.939; LSD Posthoc: AE=AC, p=0.188; AE=OC, p=0.990; AE=OE, p=0.132; AC=OC, p=0.150; OE>AC, p=0.004, OE=OC, p=0.102]

Akt

There was no difference between any of the groups in normalized Akt (p=0.765), normalized p(THR308)-Akt (p=0.267), or in the ratio of p-Akt/Akt, (p=0.305) (no figure representation). [One-Way ANOVA]

Supplemental Table Legends

Table S1 Animal Characteristics Symbols: ME Age=main effect of age, ME Trained=main effect of training, ME Inter.=interaction effect of age*training (numbers in columns=p-value from 2x2 ANOVA with bold indicating significance); different letters indicate differences at p<0.10; " ǂ "≠OC, p<0.05; " § "≠AE, p<0.05; " ¥ "=AE; BMI=body mass index; sac.=sacrifice; gbm=grams of body mass; PCSA=physiological cross sectional area; P_0=maximum tetanic force; g=grams; mg= milligram; mN=milliNewton; kg/m^2=kilogram divided by meters squared .

Table S2 Velocity of Contraction Data presented as means ± standard error. **Symbols**: AC=adult control, AE=adult exercise, OC=old control, OE=old exercise, different letters indicate differences at p<0.10; " ǂ "≠OC, p<0.05; " § "≠AE, p<0.05; " ¥ "=AE; Age=main effect of age, Training=main effect of training; Inter.=interaction of age*training, p-value, from 2x2 ANOVA, bold indicating significance; fl/s=fiber lengths/sec.

Table S3 Power Production Data presented as means ± standard error. **Symbols**: P_{max}=maximum power output, %P_0@Pmax=the percentage of P_0 (maximum force) where P_{max} occurs; AC=adult control, AE=adult exercise, OC=old control, OE=old exercise, different letters indicate differences at p<0.10; " ǂ "≠ OC, p<0.05; " § "≠AE, p<0.05; " ¥ "=AE; ME Age=main effect of age, ME Training=main effect of training, Inter.=interaction of age*training, numbers are p-values from 2x2 ANOVA, bold highlighting p<0.10; mN*fl/s=milliNewtons*fiber lengths/sec; [1]n=7 for OE.

Supplemental Figure Legends

Figure S1 Rotarod A. Rotarod Difference in Seconds Means of all four groups. **B. Rotarod Percent Change** Means of all four groups. **C. Regression** Equation is simple linear regression of rotarod difference in seconds (y value) in respect to mass of mouse in grams at sacrifice (x-value). **D. Rotarod Main Effect of Training** From 2x2 ANOVA **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at p<0.05 and "#" indicates a trend of p<0.10; lines delineate significance.

Figure S2 Grip Test A. Grip Test Difference in Seconds Means of all four groups. **B. Grip Test Percent Change** Means of all four groups. **C. Regression** Equation is simple linear regression of grip test difference in seconds (y value) in respect to mass of mouse in grams at sacrifice (x-value). **D. Grip Test Main Effect of Training** From 2x2 ANOVA **Symbols:** AC= adult control, AE= adult exercise, OC=old control, OE=old exercise; s=seconds, g=grams, number at base in bar graphs=means; "*" indicates significance at p<0.05 and "#" indicates a trend of p<0.10; lines delineate significance.

Figure S3 Training Physiology A. Normalized Training Force Equation is simple linear regression of training force normalized to body mass (y value) in respect to the training session (x-value). **B. Training Power B.** Equation: Simple linear regression, y=power and x=number of the training session in which the data was recorded. **C. Normalized Training Power** Equation: Simple linear regression, y=normalized power and x=number of the training session in which the data was recorded. **Symbols:** Symbols: diamonds=AE=adult exercise (n=7), squares=OE=old exercise (n=6); mN=milliNewtons, gbm=grams of body mass; mW=milliWatts; mW/gbm= milliWatts per gram of body mass.

Figure S4 Contractile Force A. P_0 All 4 group means. **B. P_0 Normalized to Body Mass** All 4 group means. **C. Regression of P_0 with respect to Body Mass** Equation: simple linear regression y=P_0 and x=body mass at sacrifice. **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at p<0.05 and "#" indicates a trend of p<0.10; lines delineate significance; mN=milliNewtons, gbm=grams of body mass; number at base of bar graphs=means.

Figure S5 Contractile Velocity A. Force-Velocity Curve B. Expanded view 0-40%P_0 C. Expanded View 40-100%P_0 Symbols: AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; fl/s=fiber lengths per second; %P_0=percentage of maximum isometric tetanic force.

Figure S6 Contractile Power Symbols: AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at p<0.05; %P_0=percentage of maximum isometric tetanic force; mN*fl/s=milliNewtons (force) multiplied by fiber lengths per second (velocity of contraction).

Figure S7 SOL Wet Mass A. SOL Mass B. Normalized SOL Mass in relation to body mass**. Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at p<0.05 and "#" indicates a trend of p<0.10; lines delineate significance; mg= milligrams; gbm=grams of body mass; number in base of bars=mean.

Figure S8 Plantaris Fiber CSA A. Individual Mice Means Mean is +5% Adults, +5% in Old with exercise. AC, n=5; AE, n=4, OC, n=4; OE, n=4 **B. Pooled Fiber Means C. Adult Fiber Distribution** Histogram of Distribution of Adult CSA Also reported in Chapter 3, reprinted for comparison purposes, median +7%, significant rightward shift after exercise. **D. Old Fiber Distribution** Histogram Distribution of Old CSA, median +10%, significant rightward shift after exercise. **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; in histograms light boxes=control, dark=exercise; letters indicate significant differences; Frequency%=percentage of fibers out of the total number measured; µm²=micrometers squared, CSA=cross sectional area of plantaris fibers.

Figure S9 Anabolic Signaling A. Phosphorylated p70S6K B. p70S6K C. Representative Blots In p70S6K blot the top band is phosphorylated and the bottom band is not, both were combined for total p70 content. **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; number in bar graphs are means. Abundance is normalized to the level of the adult control group; BC=blot control, PC=positive control for p-p70S6K, NC=negative control for p-p70S6K; E=old exercise, C=old control.

Table S1 Animal Characteristics Symbols: ME Age=main effect of age, ME Trained=main effect of training, ME Inter.=interaction effect of age*training (numbers in columns=p-value from 2x2 ANOVA with bold indicating significance); different letters indicate differences at p<0.10; " ǂ "≠OC, p<0.05; " § "≠AE, p<0.05; " ¥ "=AE; BMI=body mass index; sac.=sacrifice; gbm=grams of body mass; PCSA=physiological cross sectional area; P_0=maximum tetanic force; g=grams; mg=milligram; mN=milliNewton; kg/M^2=kilogram divided by meters squared.

	Unit	Adult Control	Adult Exercise	Old Control	Old Exercise	ME Age (p)	ME Trained (p)	ME Inter. (p)
Body Mass initial	g	32.6±0.7 a	29.9±0.9 b	32.7±0.7 a	34.0±0.8 a	**0.014**	0.368	**0.018**
Body Mass at sac.	g	40.9±0.9 a	37.1±0.4 a	32.3±0.6 b	32.9±0.9 b	**0.001**	0.359	0.197
Body Mass change	%	24.7±4.0 a	24.4±11.6 a	-1.4±2.5 b	-3.3±2.8 b	**<0.001**	0.765	0.816
BMI at sac.	kg/m^2	4.0±0.1 a	3.7±0.1 a	3.3±0.1 b	3.5±0.1 b	**0.001**	0.853	**0.041**
SOL Mass*	mg	12.6±0.7 a	14.5±0.8 b	9.5±0.6 c	10.9±0.5 c ǂ	**<0.001**	**0.016**	0.134
SOL Mass/ gbm	mg/ gbm	0.31±0.01 a	0.39±0.01 b	0.30±0.02 a §	0.32±0.03 a	0.160	**0.009**	0.465
SOL Fiber Length	mm	8.2±0.2 a	8.7±0.2 a	7.8±0.2 a §	8.4±0.1 a ǂ	**0.093**	**0.020**	0.771
SOL PCSA	mm^2	1.00±0.03 a	1.10±0.06 a	0.80±0.04 b	0.96±0.04 b	**<0.001**	**0.088**	0.661
P_0	mN	246.9±12.9 a	249.5±9.9 a	178.2±9.7 c §	184.6±7.3 c §	**<0.001**	0.440	0.583
P_0/gbm	mN/ gbm	7.6±0.3 a	8.4±0.3 b	5.4±0.3 c §	5.8±0.3 c §	**<0.001**	**0.071**	0.473

Table S2 Velocity of Contraction Data presented as means ± standard error. **Symbols**: AC=adult control, AE=adult exercise, OC=old control, OE=old exercise, different letters indicate differences at p<0.10; " ǂ "≠ OC, p<0.05; " § "≠AE, p<0.05; " ¥ "=AE; Age=main effect of age, Training=main effect of training; Inter.=interaction of age*training, p-value, from 2x2 ANOVA, bold indicating significance; fl/s=fiber lengths/sec.

Velocity fl/s	AC	AE	OC	OE	2x2 ANOVA p-value	Age	Training	Inter.
V_{max}	4.5±0.3 a	4.2±0.2 a	3.7±0.2 b¥	3.7±0.2 b¥	**<0.001**	**0.014**	0.549	0.488
10%P_0	2.44±0.096 a	2.59±0.139 a	2.14±0.112 b	2.17±0.116 a	**<0.001**	**0.005**	0.447	0.618
20%P_0	1.61±0.061 a	1.74±0.094 a	1.43±0.078 b	1.45±0.071 a§	**<0.001**	**0.006**	0.360	0.515
30%P_0	1.13±0.041 a	1.24±0.068 a	0.99±0.052 b	1.02±0.051 a§	**<0.001**	**0.002**	0.214	0.479
40%P_0	0.81±0.029 a	0.90±0.050 a	0.71±0.038 b	0.73±0.036 a§	**<0.001**	**0.001**	0.155	0.363
50%P_0	0.57±0.020 a	0.65±0.038 b	0.49±0.027 c	0.51±0.026 a§	**<0.001**	**<0.001**	**0.098**	0.288
60%P_0	0.39±0.016 a	0.46±0.028 b	0.33±0.018 c	0.34±0.018 c	**<0.001**	**<0.001**	**0.046**	0.224
80%P_0	0.13±0.011 a	0.18±0.015 b	0.10±0.008 c	0.11±0.008 a	**<0.001**	**<0.001**	**0.009**	**0.087**
90%P_0	0.048 ±0.006 a	0.069 ±0.008 b	0.032 ±0.005 c	0.031 ±0.006 c	**<0.001**	**<0.001**	0.115	**0.085**
a/P_0 x 10^2	2.3±0.2 a	1.8±0.1 b	2.7±0.2 c	2.3±0.1 a	**<0.001**	**<0.001**	**0.008**	0.987

Table S3 Power Production Data presented as means ± standard error. **Symbols**: P_{max}=maximum power output, $\%P_0$@Pmax=the percentage of P_0 (maximum force) where P_{max} occurs; AC=adult control, AE=adult exercise, OC=old control, OE=old exercise, different letters indicate differences at p<0.10; " ǂ "≠ OC, p<0.05; " § "≠AE, p<0.05; " ¥ "=AE; ME Age=main effect of age, ME Training= main effect of training, Inter.=interaction of age*training, numbers are p-values from 2x2 ANOVA, bold highlighting p<0.10; mN*fl/s=milliNewtons*fiber lengths/sec; [1]n=7 for OE

Power mN*fl/s	**AC n=9**	**AE n=6**	**OC n=12**	**OE n=9**	**2x2 ANOVA p-value**	**ME Age**	**ME Training**	**Inter.**
P_{max} [1]	89.1±4.7 a	97.7±10.5 a	54.7±5.3 b	61.9±46.5 b	**<0.001**	**<0.001**	0.240	0.918
10%P_0	61.5±4.0 a	65.1±5.5 a	42.7±3.7 b	42.7±4.0 b	**<0.001**	**<0.001**	0.396	0.983
20%P_0	81.1±5.0 a	87.2±7.3 a	53.0±5.1 b	56.8±4.8 b	**<0.001**	**<0.001**	0.382	0.845
30%P_0	85.6±5.2 a	93.5±7.7 a	54.1±5.2 b	59.9±5.0 b	**<0.001**	**<0.001**	0.245	0.860
40%P_0	81.6±4.8 a	90.7±7.5 a	51.3±5.0 b	56.8±4.7 b	**<0.001**	**<0.001**	0.196	0.743
50%P_0	72.1±4.2 a	81.9±6.9 a	44.6±4.5 b	49.7±4.1 b	**<0.001**	**<0.001**	0.144	0.635
60%P_0	59.9±3.4 a	69.1±6.1 a	35.5±3.7 b	40.0±3.3 b	**<0.001**	**<0.001**	**0.081**	0.501
80%P_0	26.1±1.7 a	36.3±3.9 b	14.5±1.8 c	17.2±1.5 c	**<0.001**	**<0.001**	**0.005**	0.217
90%P_0	10.6±1.2 a	15.5±2.1 b	5.5±1.1 c	5.4±1.0 c	**<0.001**	**<0.001**	**0.078**	**0.070**
$\%P_0$ at P_{max} [1] (%)	27.3±0.6 a	29.1±0.5 b	27.3±0.4 c	27.9±0.3 c	**<0.001**	0.188	**0.022**	0.188

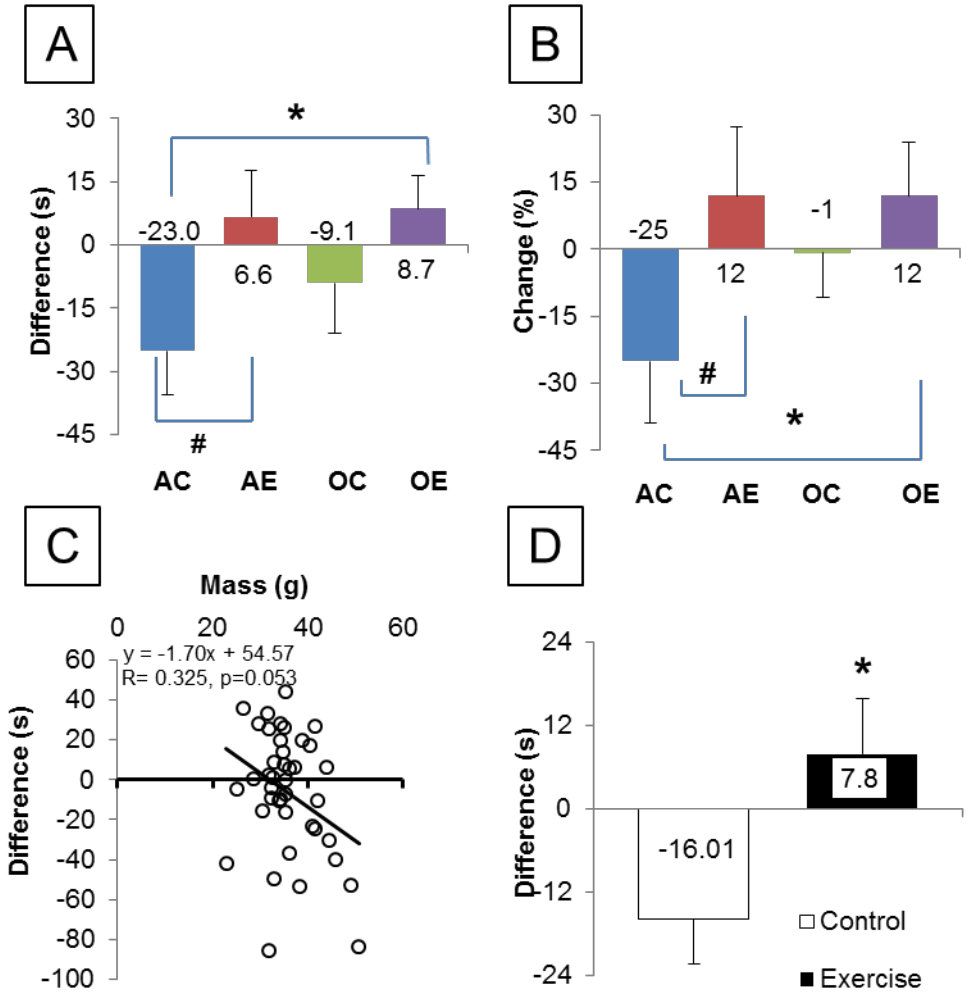

Figure S1 Rotarod Improves with Training A. Rotarod Difference in Seconds Means of all four groups. **B. Rotarod Percent Change** Means of all four groups. **C. Mass and Rotarod Difference Unrelated** Equation is simple linear regression of rotarod difference in seconds (y value) in respect to mass of mouse in grams at sacrifice (x-value). P-value is non-significant. **D. Rotarod Main Effect of Training** From 2x2 ANOVA **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at $p<0.05$ and "#" indicates a trend of $p<0.10$; lines delineate significance.

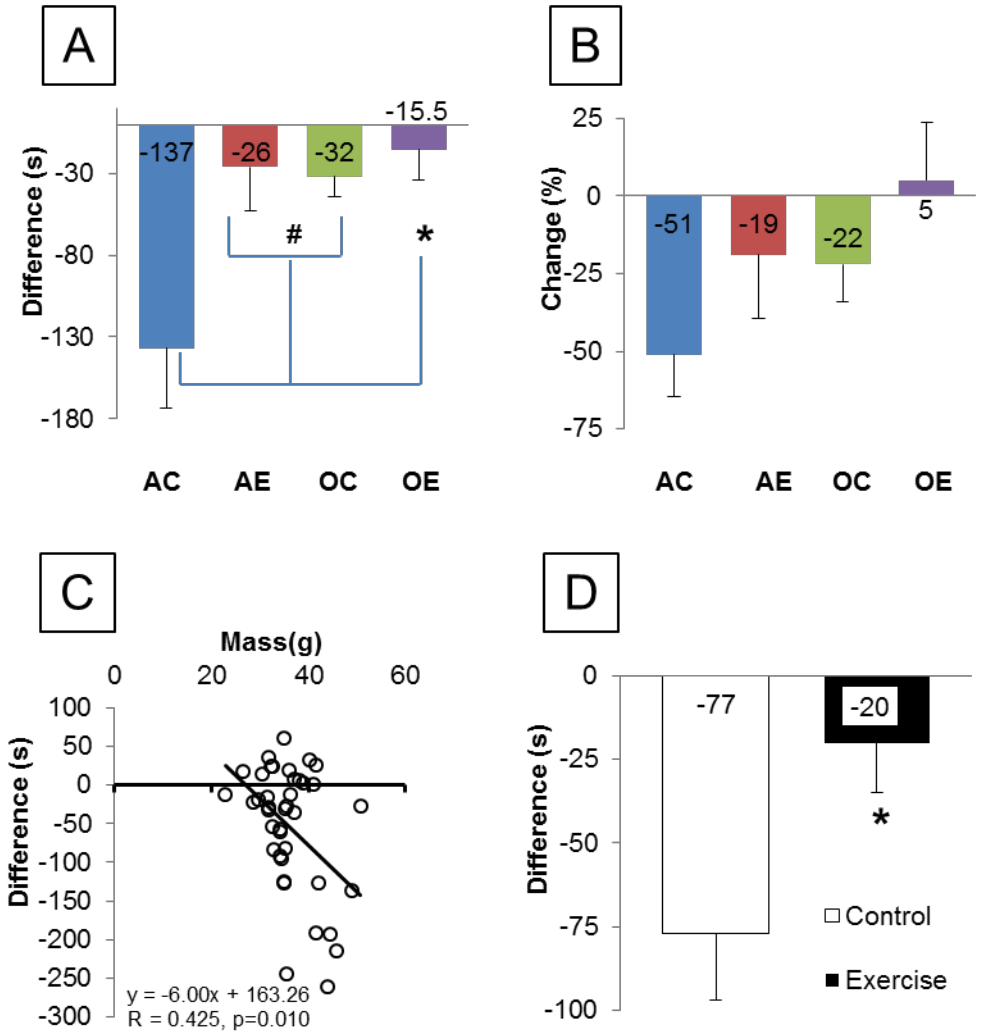

Figure S2 Grip Test A. Grip Test Difference in Seconds Means of all four groups. **B. Grip Test Percent Change** Means of all four groups. **C. Regression shows Relationship of Mass and Grip Difference** Equation is simple linear regression of grip test difference in seconds (y value) in respect to mass of mouse in grams at sacrifice (x-value). **D. Grip Test Main Effect of Training** From 2x2 ANOVA **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; s=seconds, number at base in bar graphs=means; "*" indicates significance at $p<0.05$ and "#" indicates a trend of $p<0.10$; lines delineate significance.

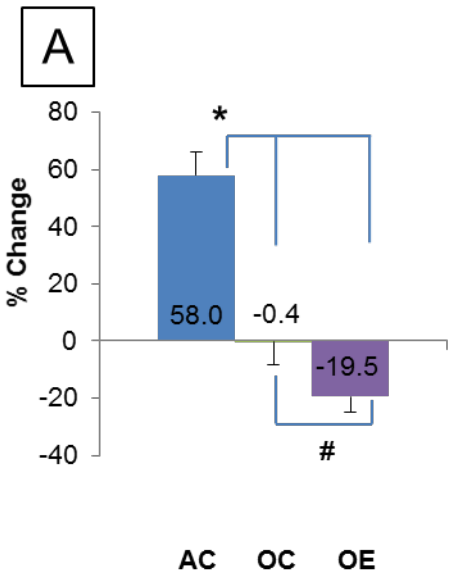

Figure S3 Body Composition improves with Training (means) Symbols: AC=adult control (n=12), OC= old controls (n=7) and OE= old exercise mice (n=11); number at base in bar graphs =means; "*" indicates significance at p<0.05 and "#" indicates a trend of p<0.10. Lines indicate significance. [1-way ANOVA f=24.403, p<0.001. AC>OC, p<.001; AC>OE, p<0.001; OC>OE, p=0.072].

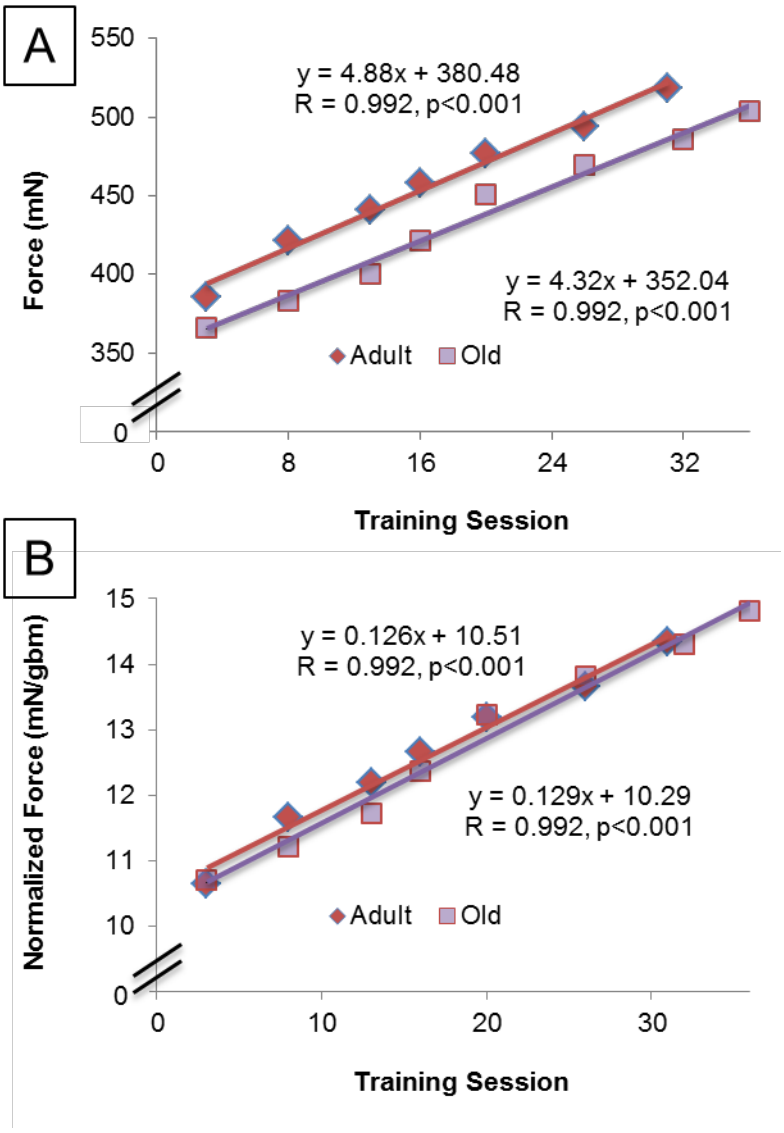

Figure S4 Training Force A. Training Force over Training Period Equation is simple linear regression of training force (y value) in respect to the training session (x-value). Regressions significantly different with the mean increase (adjusted) of 7.6% with training in the adult group [Univariate GLM ANCOVA, adjusted for training session, F=368, p<0.001; Main Effect of Age group, p<0.001, training session as adjustor, p<0.001] **B. Normalized Training Force** Equation is simple linear regression of training force normalized to body mass (y value) in respect to the training session (x-value). Trend for Regressions to be different, with the mean increase (adjusted) of 1.4% with training in the adult mice (Univariate GLM ANCOVA, adjusted for training session, F=361, p<0.001; Main Effect of Age group, p=0.095, training session as adjustor, p<0.001) **Symbols:** Symbols: diamonds=AE=adult exercise (n=7), squares=OE=old exercise (n=6); mN=milliNewtons, gbm=grams of body mass.

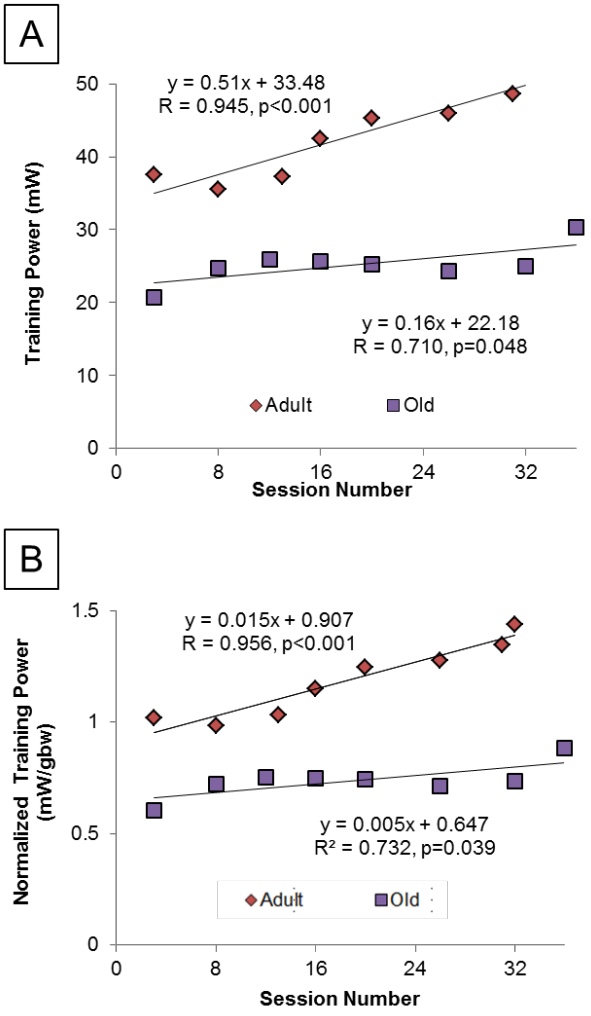

Figure S5 Training Power A. Training Power B. Equation: Simple linear regression, y=power and x=number of the training session in which the data was recorded. **Normalized Training Power** Equation: Simple linear regression, y=normalized power and x=number of the training session in which the data was recorded. **Symbols:** Adult=diamonds=adult exercise, Old=squares=old exercise; mW=milliWatts; mW/gbm=milliWatts per gram of body mass.

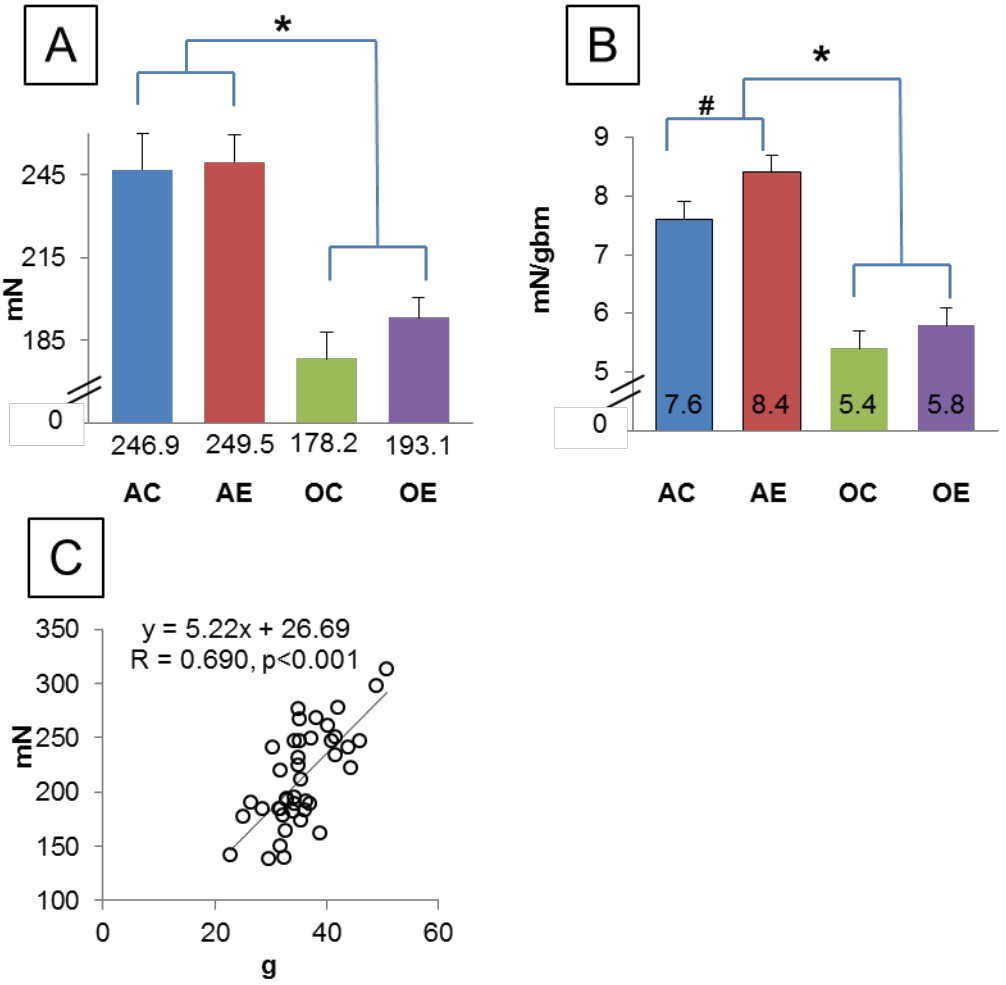

Figure S6 Contractile Force A. P_0 All 4 group means. **C. P_0 Normalized to Body Mass** All 4 group means. **C. Regression of P_0 with respect to Body Mass** Equation: simple linear regression $y=P_0$ and x=body mass at sacrifice. **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at $p<0.05$ and "#" indicates a trend of $p<0.10$; lines delineate significance; mN=milliNewtons, gbm=grams of body mass; number at base of bar graphs=means.

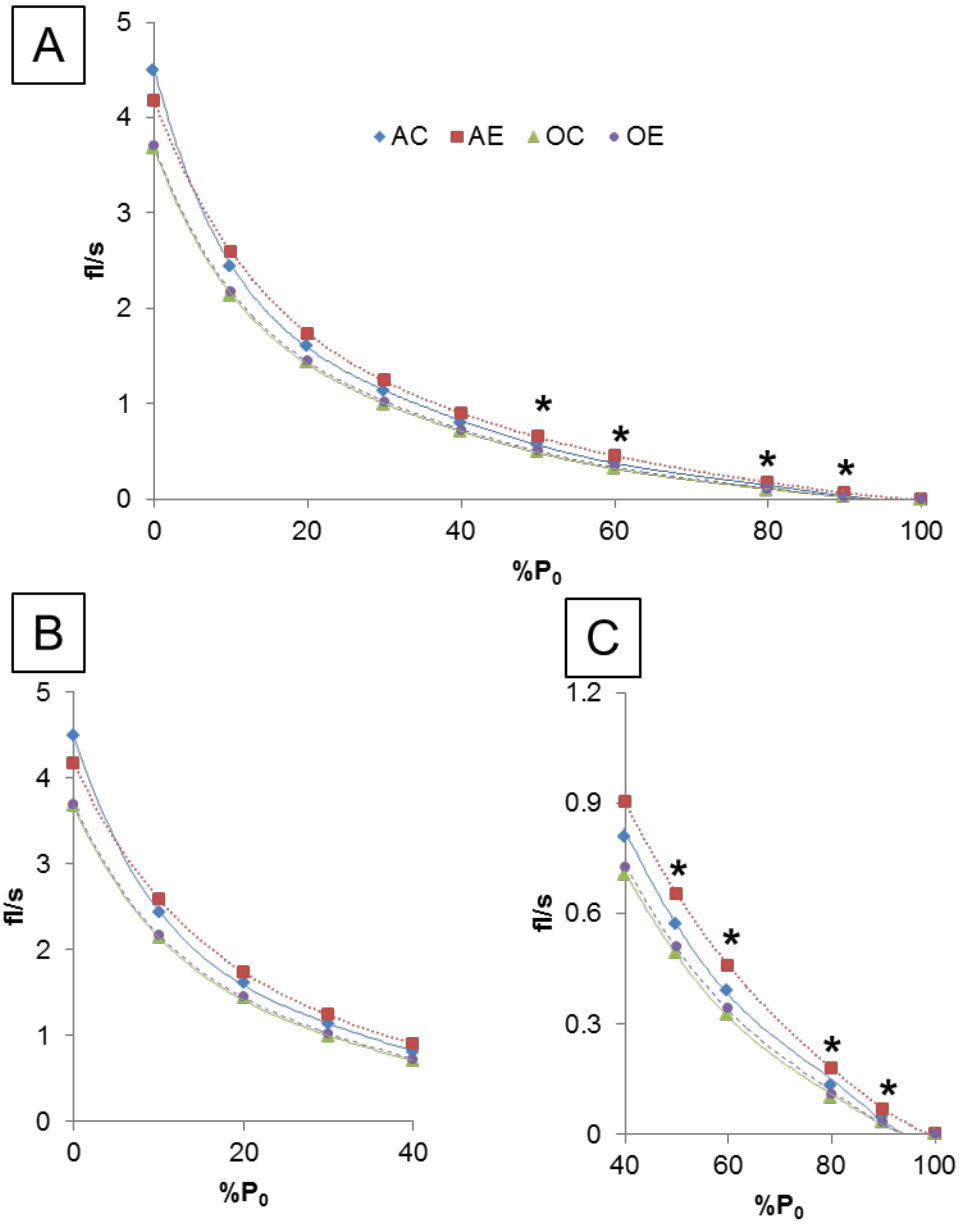

Figure S7 Contractile Velocity A. Force-Velocity Curve AC group significantly increased at velocities measured at 50% of P_0 and higher. OE is not different from OC. Age related differences are at all velocities. **B. Expanded view 0-40%P_0 C. Expanded View 40-100%P_0 Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; fl/s=fiber lengths per second; %P_0=percentage of maximum isometric tetanic force.

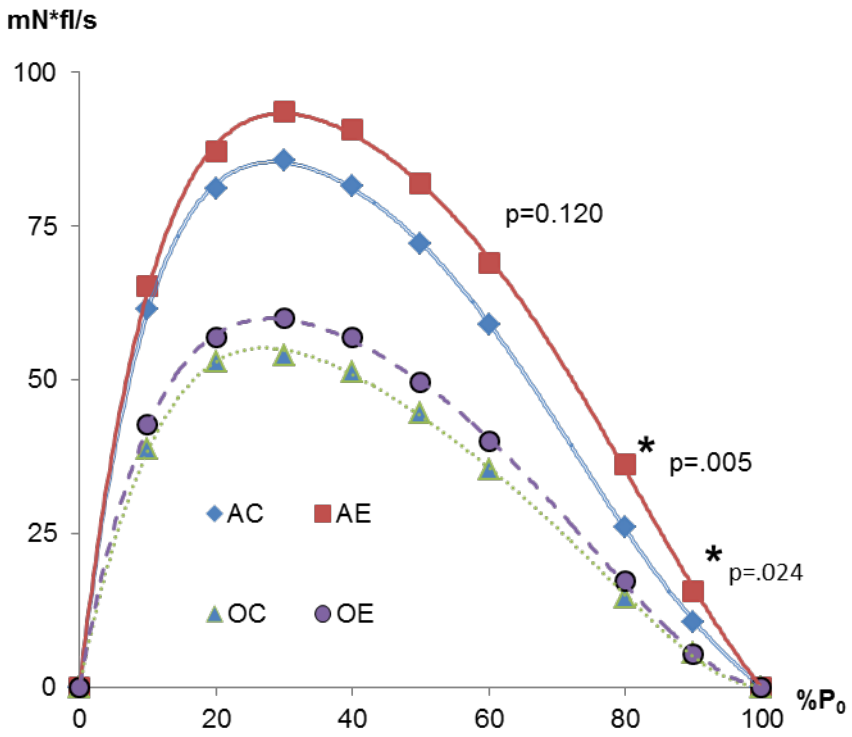

Figure S8 Contractile Power output is increased in AE at 80 and 90% P_0. **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at $p<0.05$; $\%P_0$=percentage of maximum isometric tetanic force; mN*fl/s=milliNewtons (force) multiplied by fiber lengths per second (velocity of contraction).

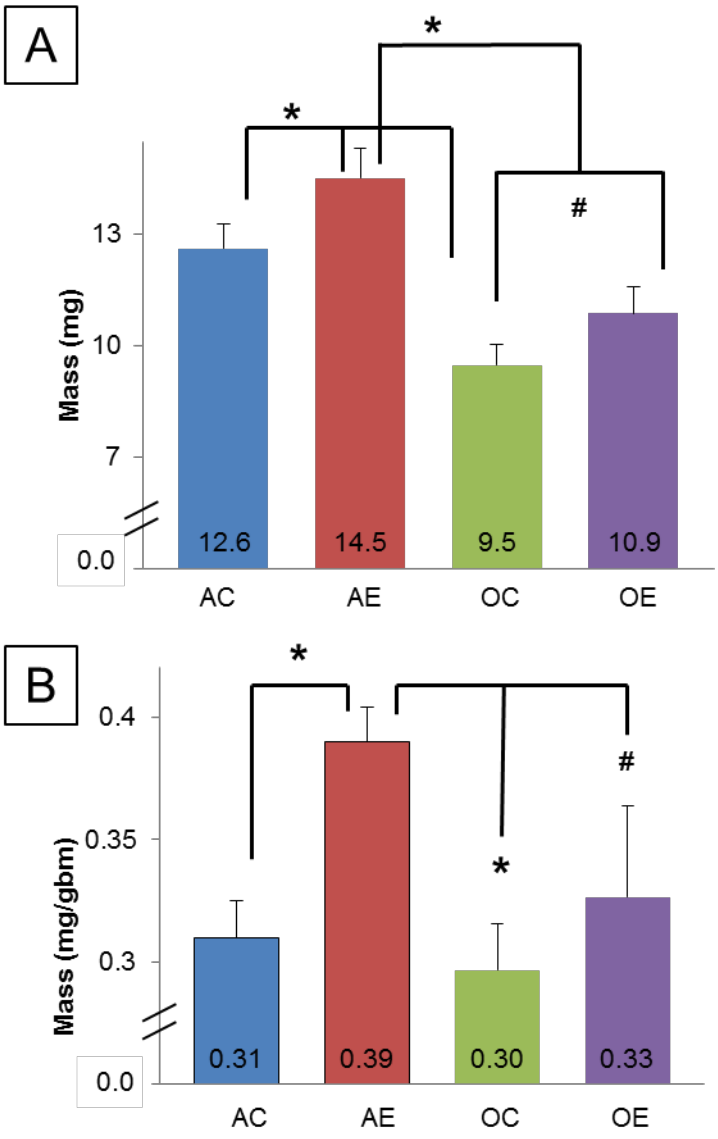

Figure S9 SOL Wet Mass A. SOL Mass Increased with Training [2x2 ANOVA F=10.271, p<0.001 Main Effects: Age, p<0.001; Training, p=0.014; age*training interaction: p=0.617; LSD Posthoc: AE (n=6) Soleus 15%>AC (n=13) , p=0.019; AC>OC (n=12) p=0.001; AC=OE (n=7) p=0.159; AE>OC, p<0.001; AE>OE, p=0.002; % change with training (main effect)= 18.2%, % change with aging (main effect)= 23.7%] **B. Normalized SOL Mass Increased with Training** Mass of the SOL in relation to body mass**.** [2x2 ANOVA F=3.053, p=0.042 Main Effects: Age, p=0.077; Training, p=0.019; age*training interaction: p=0.279; LSD Posthoc: AE 26%>AC, p=0.021; AE>OC, p=0.007; AE>OE, p=0.085; AC=OC, p=00.566; AC=AE, p=0.637; % change with training (main effect)= 16.8%, % change with aging (main effect)= -8.7%] **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; "*" indicates significance at p<0.05 and "#" indicates a trend of p<0.10; lines delineate significance; mg=milligrams; gbm=grams of body mass; number in base of bars=mean.

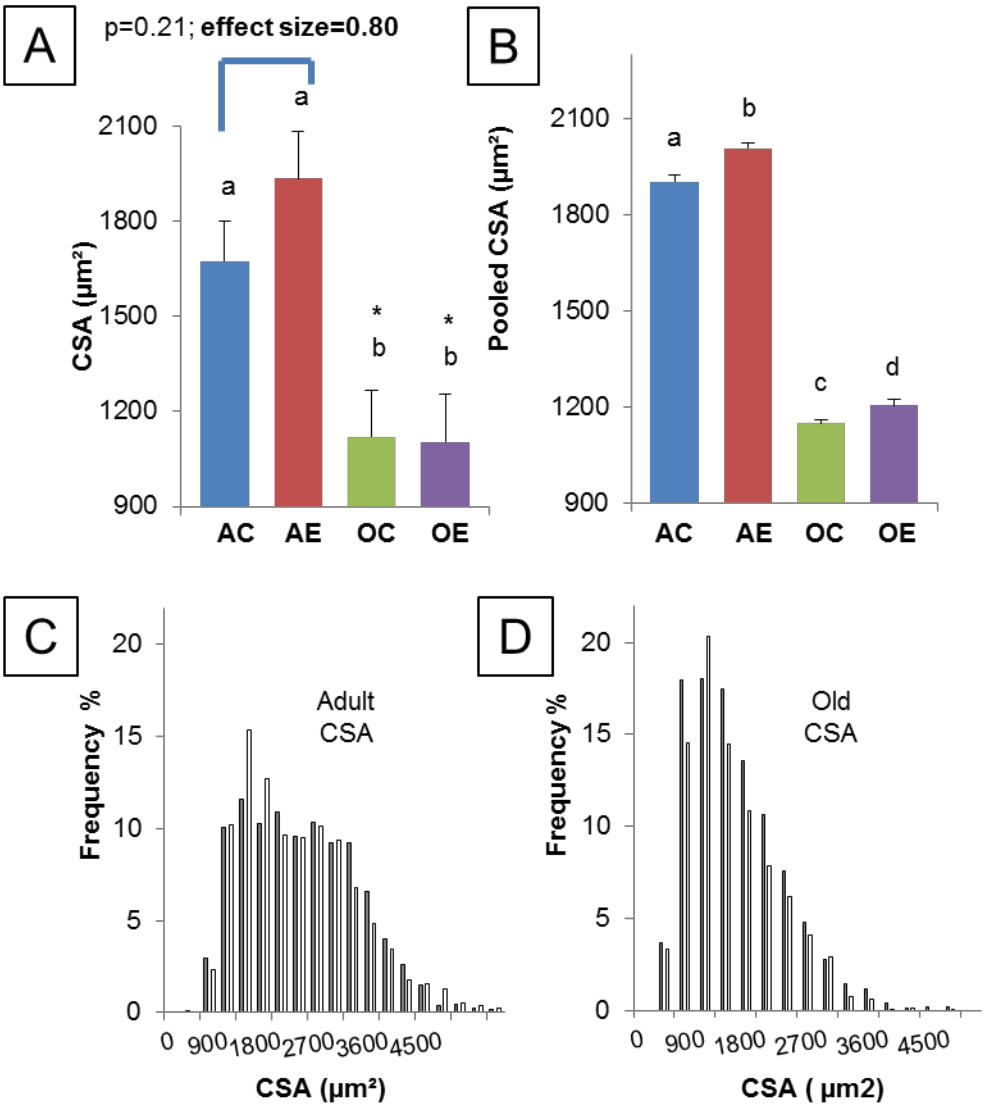

Figure S10 Plantaris Fiber CSA A. Individual Mice Means Mean is +5% Adults, +5% in Old with exercise. AC, n=5; AE, n=4; OC, n=4; OE, n=4 **B. Pooled Fiber Means C. Adult Fiber Distribution** Histogram of Distribution of Adult CSA Also reported in Chapter 3, reprinted for comparison purposes, median +7%, significant rightward shift after exercise. **D. Old Fiber Distribution** Histogram Distribution of Old CSA, median +10%, significant rightward shift after exercise. **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; in histograms light boxes=control, dark=exercise; letters indicate significant differences; Frequency%=percentage of fibers out of the total number measured; µm²=micrometers squared, CSA=cross sectional area of plantaris fibers

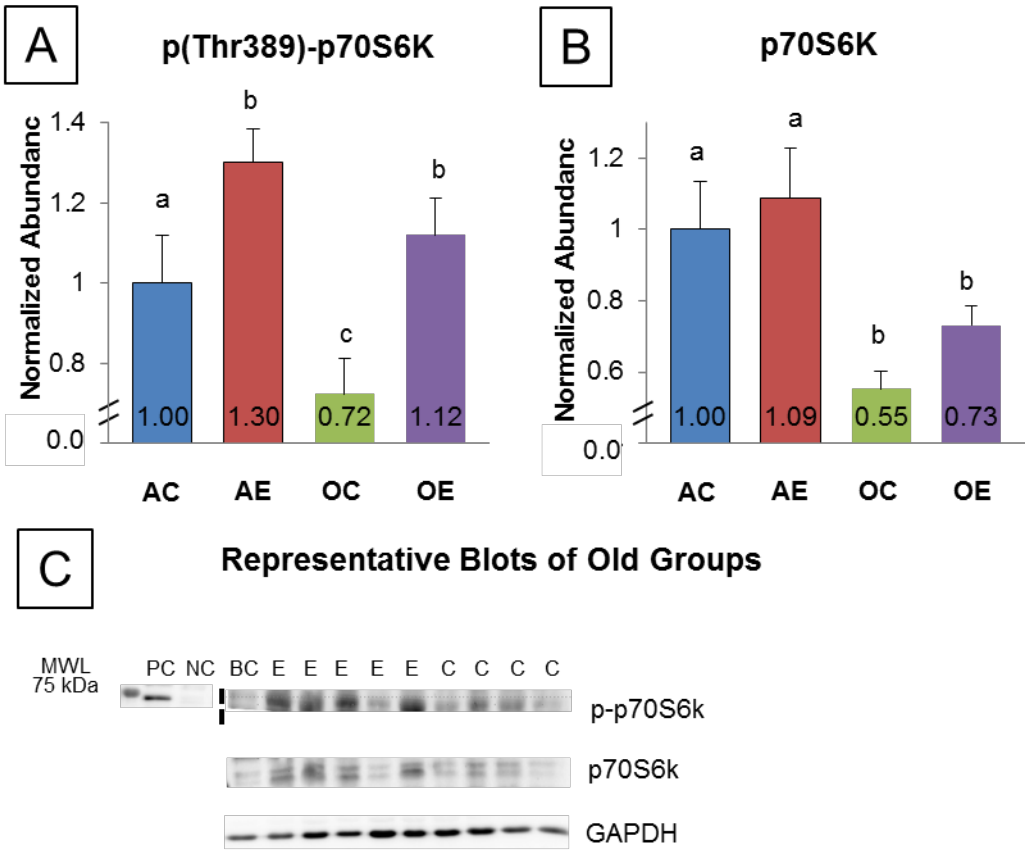

Figure S11 Anabolic Signaling A. Phosphorylated p70S6K B. p70S6K C. Representative Blots In p70S6K blot the top band is phosphorylated and the bottom band is not, both were combined for total p70 content. **Symbols:** AC=adult control, AE=adult exercise, OC=old control, OE=old exercise; number in bar graphs are means; error bar=standard error; Abundance is normalized to the level of the adult control group; BC=blot control, PC=positive control for p-p70S6K, NC=negative control for p-p70S6K; E=old exercise, C=old control; different letters indicate significance.

Epilogue: Conclusion and Summary

Sarcopenia, the age-related loss of muscle mass and strength, is an important health concern that affects large numbers of older adults. Sarcopenia can lead to a lower quality of life, poor prognoses after procedures, low gait speed, functional disability, difficulties with activities of daily living, increased incidence of falls, increased mortality, development of frailty, and the eventual loss of independence. To date, even after much study the exact etiology of sarcopenia has not been identified, though there are many and varied sources that do contribute at least partly to the development and progression of the condition. In addition, there is no cure.

Though there is no cure for sarcopenia, exercise has much promise as a countermeasure. Resistance training, in particular, increases muscle mass and strength, and improves functional ability in adults of any age. One challenge in the search for treatment strategies to mitigate sarcopenia is the blunting of response to anabolic stimuli often observed in the older adult population. The term for this is age-related anabolic resistance. Thus, not only does muscle mass and strength decline with age, but the response to anabolic stimuli also declines in effectiveness.

Animal models are the first step in investigating both mechanisms and for the first trial of novel treatment strategies, particularly pharmaceutical. This thesis

focused on describing and developing a mouse model that will be used to investigate treatments for sarcopenia, synergistic effects of multiple treatment strategies to overcome anabolic resistance, and basic biology of exercise. As the first step, assessment strategies and tools were developed and the mouse model baseline values evaluated. Second, a mouse model of resistance training was created and validated. Finally, the resistance training protocol was tested in an older cohort of mice to determine evidence of anabolic resistance.

Collectively, Chapters 2 and 3 present a story where functional ability and contractile parameters decline with age. Functional and contractile ability was improved through resistance exercise, as described in Chapter 4. Chapter 5 demonstrated that older mice improved in some parameters after undergoing exercise training, sometimes to the same degree as in adults. However, in other parameters the degree of improvement was blunted in the older, or in some cases insignificant. Thus, a stimulus sufficient to improve outcomes in the adult mice does not always produce equivalent results in the older mice.

Interventions with longer duration and/or increased intensity may lead to better adaptations in the older mice; however, it is also possible that older mice simply may not have the ability to respond to anabolic stimuli younger mice. Thus, investigating the etiology of anabolic resistance by examining age-associated phenotypic differences at the cellular and systemic level might lead to strategies

that ultimately could be combined to work in synergy with exercise training to improve both anabolic response and natural repair/rehabilitation mechanisms. Future studies will address these questions.

Individual variability in outcome measurements is a glaring confounder in aging studies. One early goal of this thesis work was to build a mathematical construct that would serve to reduce the effect of the variability on detecting changes in means. The "*C57BL/6 Neuromuscular Healthspan Scoring System",* a composite scoring system described in Chapter 2, is an important tool that can be used to delineate changes in neuromuscular health after intervention with a much greater power than the individual outcome measurements (rotarod, grip test, and maximum isometric force of the EDL muscle) that make up the composite can alone. The strategy used to produce the score can easily be adapted to other types of measurements by substituting/adding validated outcomes to the composite of scores. Chapter 2 adds not only this valuable tool to the literature, but also for the first time provides a linear regression analysis of functional ability and contraction across the lifespan of the C57BL/6 mouse.

Chapter 2 elucidated functional loss in the aging mouse and gave insight in loss of contractile force in the EDL muscle. In Chapter 3 "*C57BL/6 Lifespan Study: Age-Related Declines in Muscle Power Production and Contractile Velocity*", the focus was on investigating changes over the lifespan in velocity of contraction

and power production in both the EDL and soleus muscles along the entire continuum of concentric contraction. Previous literature examined contractile velocity function below 50% of maximum isometric force (P_0) mainly in order determine unloaded maximum velocity. Likewise, many prior investigations into power production concentrated on maximum power (around 30-40% P_0), not power over the entire force-power curve.

Movement in life requires contraction over the entire range of the force-velocity and force-power curves. Thus, for the first time, comprehensive results encompassing the entire range of contraction over the lifespan of the mouse are reported. Data from Chapter 3 describes both higher levels of concentric contraction (60, 80 and 90% P_0) and also regresses the results over the mouse adult lifespan from 6-32 months of age. The data strongly suggests that age more prominently affects concentric contraction at more difficult tasks, i.e. at loads above 50% P_0.

At this point the stage was set for the creation of a protocol in the mouse model that would serve as a mimetic for human resistance exercise studies. Therefore, Chapter 4, "*Voluntary Resistance Training in Adult Mice*" describes and validates a training system that serves as a mouse model mimetic for human resistance exercise. A number of outcome measurements were chosen in order to assess whether or not the training protocol (designed utilizing human principles of weight

training) would result in adaptations in the mice similar to what would be expected in humans. Indeed, the preponderance of evidence suggested adaptations occurred, thus validating the protocol. Prior to this study there was no such mimetic of human voluntary weight lifting in the mouse.

Finally, Chapter 5, *"Voluntary Resistance Training in Elderly Mice and Signs of Anabolic Resistance",* applied the protocol designed and validated in Chapter 4 to an aged cohort of mice. The mice that underwent training in Chapter 4 were 12 months of age (100% survival) at the study completion, whereas the older cohort of mice was 28 months of age at the end of their training (50% survival). In human terms, the younger adult mice were the equivalent of 30 year olds and the older adult mice were like 78-80 year olds. Adaptations occurred in the older mice, but the adaptations were not as extensive as those in the younger. These findings were consistent with the well-established concept of anabolic resistance in older individuals. More importantly, the results confirm the strength of this model as an exceptional tool to investigate anabolic resistance. Future directions using this model include investigating the synergy of other interventions (whether pharmaceutical, nutritional, or hormonal) with resistance training to determine strategies to overcome age-related anabolic resistance. In addition, basic exercise biology mechanism research will benefit from have this novel model of voluntary resistance exercise in the mouse available. Overall this thesis has

added valuable research tools and information to the body of literature in the field that will enable future research to progress better as a result of our efforts.

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